The behaviour of iodine in the terrestrial environment. An investigation of the possible enzymatically controlled iodination of humic acid

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An Investigation of the Possible Enzymatically Controlled Iodination of Humic Acid

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February 1990

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THE BEHAVIOUR OF IODINE IN THE TERRESTRIAL ENVIRONMENT. AN INVESTIGATION OF THE POSSIBLE ENZYMATICALLY CONTROLLED IODINATION OF HUMIC ACID

Jesper V. Christiansen

Abstract

Literature on the geochemistry of iodine is surveyed, focusing on fundamental chemical aspects which influence the migration behaviour of iodine in the terrestrial environment. It is stated that the organic fraction in soil plays the predominant role in the retention of iodine. Simple aromatic molecules serve as simple models for humic acid, and humic acid is iodinated catalyzed by haloperoxidases. The enzymatically controlled iodination of humic acid is described in detail and it is demonstrated that the results may reflect a kind of equilibrium. It is shown that soil extracts are able to catalyze the iodination of humic acid and it is suggested that extracellular peroxidases in soil are responsible for the reaction. The enzymatically controlled iodination of humic acid is discussed and some considerations about the influence on the migration of iodine in the terrestrial environment are given.

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DANSK RESUME

Jod er nr. 46 i rækken af de almindeligste grundstoffer på jorden. Det er essentielt for mennesker og pattedyr, og har af den årsag været genstand for mange undersøgelser, som skulle belyse dets globale cyklus såvel som dele heraf. I nyere tid har en radioaktiv jodisotop ^{129}I, af helsemæssige årsager, tiltrukket sig opmærksomhed. På den baggrund er det hensigten med denne rapport at belyse fundamentale kemiske aspekter, som påvirker jods spredning i jord -og grundvandssystemer.

Det er tidligere sandsynliggjort, at jod binder til den organiske fraktion i jord, og det er foreslået at extracellulære enzymer af peroxidase gruppen, er ansvarlige for denne fixering.

For at få et basalt kendskab til den enzymkatalyserede joderingsreaktion blev phenol og andre aromatiske forbindelser forsøgt joderet med elementær jod og jodid i nærværelse af hydrogenperoxid og peroxidase. Det vises at de fleste forbindelser kan joderes v.h.a. elementær jod men kun phenol, orcinol og 3,5 di-hydroxybenzosyre kan joderes v.h.a. enzymer. Det konkluderes at forskellige aromatiske forbindelser inhiberer funktionen af peroxidase, idet phenol ikke joderes når disse aromatiske forbindelser er tilstede.

Jodering af humus syre (HA) blev også undersøgt. Det vises at mindst 3 forskellige peroxidaser er istand til at katalysere joderingen af humus syre i nærværelse af jodid og hydrogenperoxid. Joderingen af humussyre belyses nærmere, idet inkorporeringen måles som funktion af forskellige parametre f.eks. HA-konc., jodid-konc., enzyme-konc. m.m.. Der gives et forslag til en mekanisme, som involverer dannelsen af I_2 og derefter HOI, og det vises at graden af inkorporering i forskellige koncentrations-forhold mellem HA og jodid kan afspejle et ligevægtssytem. Det demonstreres endvidere at isotop udskiftning synes at spille en væsentlig rolle, når jod er tilgængelig som elementær jod eller som jodid i nærværelse af peroxidase og hydrogen-

peroxid.

Det vises, at der i jord findes en naturlig "evne" til at katalysere jodering af H.A, og at denne "evne" kan ekstraheres med en almindelig anerkendt metode til ekstrahering af peroxidaser fra jord. "Evnen" er varme følsom, og fungerer kun i nærværelse af hydrogenperoxid. Det foreslås at der er tale om extracellulære peroxidaser.

Til sidst diskuteres hvilke organiske substrukturer (sites) i humus syre, som kan være genstand for jodering, hvordan den reversible reaktion kan opfattes og hvordan migrationen af jod i jord kan påvirkes af de nævnte reaktioner.Det konkluderes at flere forskellige typer af sites synes at være tilgængelige for jodering, og at elektrofil jod-jod udskiftning formodentlig spiller en væsentlig rolle for migrationen af radiojod under naturlige forhold.

1. INTRODUCTION

Iodine is one of the less abundant elements in the environment. With an average concentration in the lithosphere of 0.3 ppm, iodine appears as number 46 among the more common elements, well below the 32 elements that constitute 96.6% of the lithosphere (Chilean Iodine Educational Bureau, 1956) (CIEB). Nevertheless, iodine appears as one of the essential elements for mammals, including humans, a fact which was recognized decades ago. Due to the widely prevalent disease goitre, a result of iodine deficiency, major efforts were devoted to studies of the distribution, concentrations and environmental cycle of iodine. In recent years, the long-lived radioactive iodine isotope, ^{129}I ($t_{1/2} = 1.56 \times 10^7$ years) has received considerable attention as a byproduct of nuclear energy production. ^{129}I released to the environment, e.g. by nuclear accidents, may be accumulated in the thyroid gland by its incorporation in the thyroid hormone, resulting in damaging internal radiation doses.

The public in general has become increasingly aware of the presence of halogenated substances, and more than 550 naturally occurring halogenated compounds are known (Siuda, 1980). Many different living organisms are responsible for the formation of these compounds: fungi, bacteria, plants, animals and humans (Niedleman and Geigert, 1986). It may be a little surprising to discover that the halogenated compounds found in soil and groundwater system are certainly not all of anthropogenic origin.

It seems reasonable that one of the mechanisms which can affect the migration of iodine is enzyme-catalyzed incorporation of iodine into humic substances (Behrens 1982, 1985 and 1986).

This report will try to elucidate the behaviour of iodine in the environment. However most attention will be paid to the fixation of iodine in the organic fraction in soil systems probably catalyzed by extracellular enzymes.

2. THE SPECIATION AND ENVIRONMENTAL CYCLE OF IODINE

2.1. The speciation of iodine

In the terrestrial environment iodine will predominantly exist as iodide, as visualized in the stability diagram (Fig. 1) based upon the data given by Bowen (1979). The dotted area corresponds to conditions to be found in groundwater (Jensen, 1982).

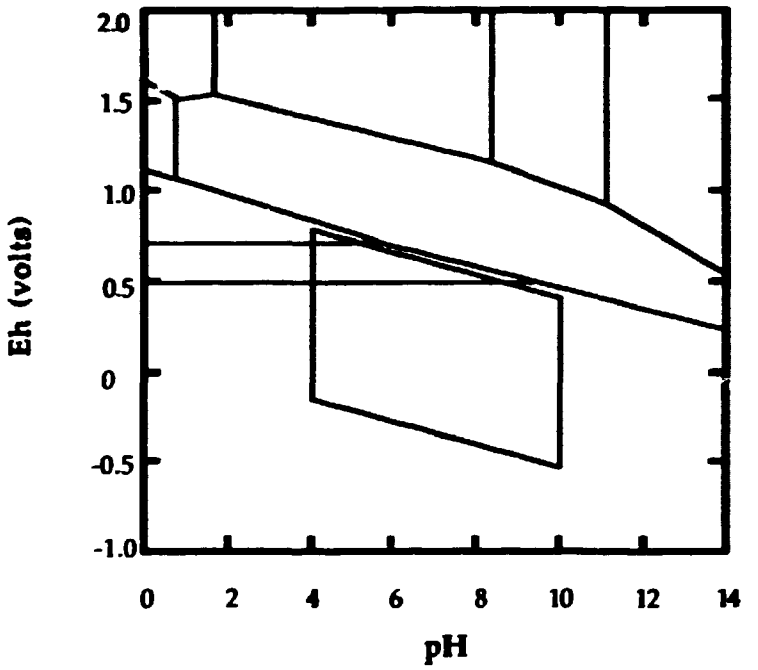


Fig. 1. Stability diagram for iodine. The dotted area corresponds to conditions to be found in groundwater.

In seawater, iodate (IO_3^-) appears to be the thermodynamically more stable iodine species. Wong and Brewer (1977) demonstrated that iodide appears only in the upper layer of seawater. Tsonogai and Sasa (1969) reported that certain organisms enzymatically are able to reduce iodate to iodide. Wong (1982) concluded that elemental iodine undergoes rapid hydrolysis to form hypoiodite (HOI) in seawater. In rainwater, Jones (1981) demonstrated that the concentrations of iodide and iodate are approximately the same.

2.2. The environmental cycle of iodine

In the course of time, several attempts to describe the global environmental cycle of iodine have appeared. In 1981 Kocher proposed a model dealing with different compartments and the mutual yearly transport, whereas a model by Whitehead (1984) takes its starting point in the different mechanisms involved in the iodine-cycle. To a first approximation, no discrepancy between the two models seems to exist, obviously owing to the fact that both models basically are founded on the same sources. A later revision of the Kocher model (White and Smith, 1984) was limited to two aspects: (a) an adjustment of the values for the transport between the single compartment, and (b) subdivision of the "soil compartment" into a solid- and a liquid part. This subdivision obviously gives a more detailed picture of the actual phenomena influencing the transport of iodine in the environment. In Fig. 2 the models proposed by Whitehead (1984) and Kocher (1981)/White and Smith (1984) are combined and further extended with some mechanistic considerations as, for instance, the involvement of enzymes in the soil compartment.

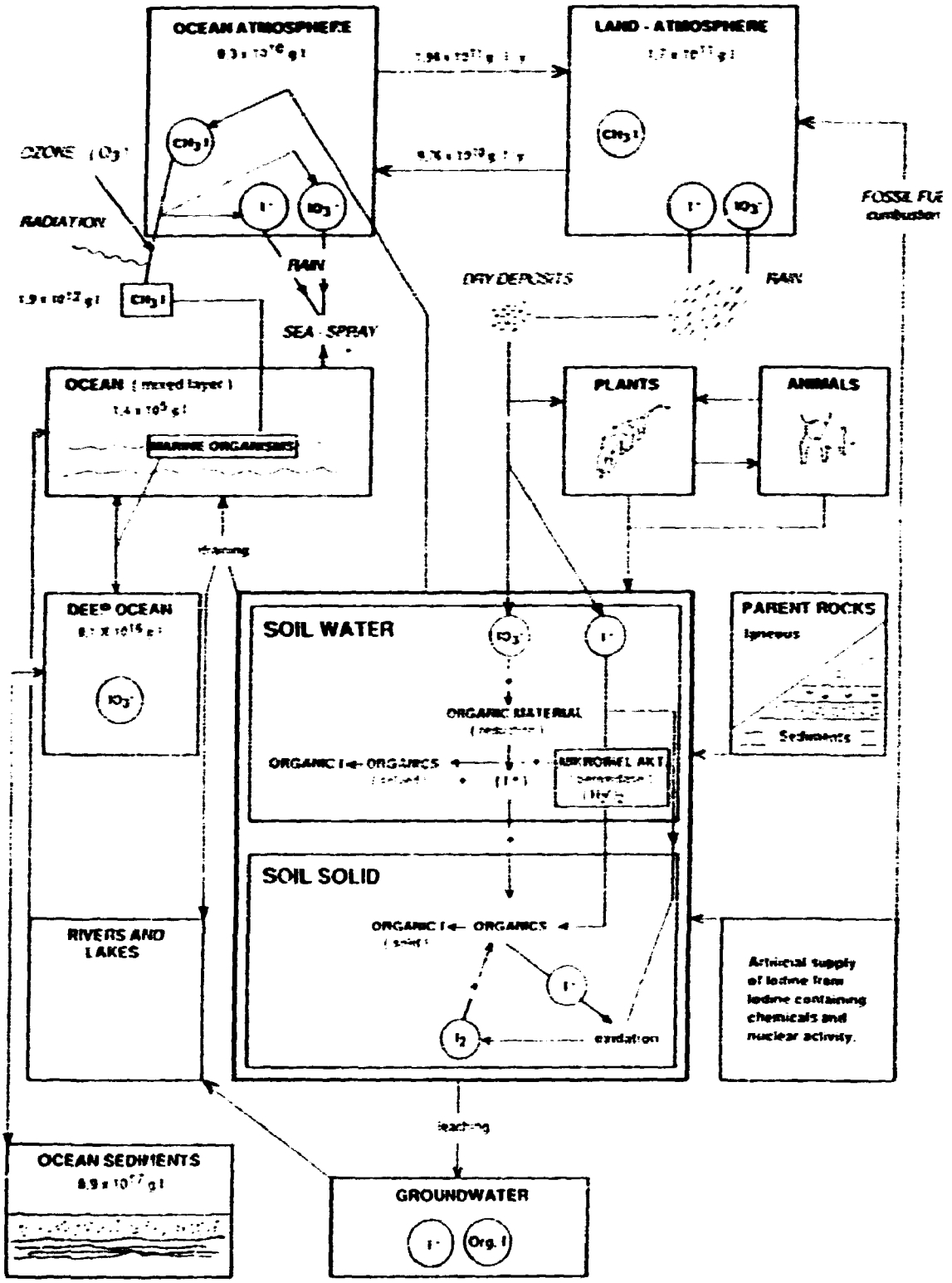


Fig. 2. The environmental cycle of iodine.

It has been suggested that iodine concentrations in soils could be related to the distance from the sea, i.e. the iodine should predominantly be supplied as a consequence of precipitation with rainwater (McClendon, 1939; Karelina, 1961). This hypothesis has, however, been rejected by Cohen (1985), who stated that the iodine concentrations for sedimentary rocks, which constitute 80% of the earth's crust, reported by Bowen (1979) merely account for the concentrations found in soils. Furthermore, Cohen (1985) questioned a long-distance transport of iodide, e.g., as adsorbed on particulate matter. He apparently disregards the fact, however, that the major transport of iodine from the sea to the atmosphere most probably takes place in the gaseous state as methyl iodide (CH_3I) (Rasmussen et al., 1982) as visualized in Fig. 2. For that reason the overall residence time of iodine in the atmosphere will probably be enhanced, whereby a more pronounced distribution appears possible. The average residence time of iodine in the atmosphere has been reported by Kocher (1981) to be approx. 15 days. The amount of methyl iodide which yearly is transformed from the sea to the atmosphere was calculated by Rasmussen et al. (1982) as approx. 1.3×10^9 kg.

CIEB (1956) found that sedimentary rocks contain iodine in significantly higher concentrations than igneous rocks; this probably can be explained by an accumulation of iodine during the sedimentation process, originating from the source rock and supply from rain as well as from, e.g., biological activity. Additionally, it can be calculated that the iodine concentration increases by a factor of ca. 20 from igneous rocks to derived soils, whereas the increase amounts to a factor of only 1-4 from sediments to derived soils.

CIEB (1956) suggested that the explanation for the latter variations may be due to some extent to the porous structure of the sediments, which may cause an enhanced leaching. It should be noted that this explanation is in contradiction with the fact that the transformation of igneous rocks to soils of necessity must involve intermediary structures of sedimentary character. Thus, the actual nature of the iodine-containing materials as well as the

conditions of the igneous rock to soil transformation most probably play a dominant role; the eventual explanation is still lacking.

Soils exhibiting high organic content typically appear to concentrate iodine in increasing amounts. Whitehead (1979) reported iodine levels up to 100 ppm for certain peaty soil samples. No clear-cut picture of the relation between iodine and organic content in soils has as yet been developed. Some authors explain this relation by a hypothesis stating that the major amount of iodine in soils originates from the atmosphere and that the environmental iodine-cycle is rather slow. Thus, "young" soils, i.e. soils developed after the last ice age have not yet obtained iodine concentrations as high as the "older" soils, despite a potentially more favourable composition (CIEB, 1956; Coldsmith, 1958). Another reasonable explanation could be the different abilities of the soil components to retain the released iodine from minerals or from an external supply. Some of these mechanisms will be dealt with in the following chapters.

3. THE INORGANIC GEOCHEMISTRY OF IODINE

Major efforts have been made to understand the behaviour of iodine/ radioiodine in the terrestrial environment. Many different minerals, igneous rocks and sedimentary rocks have been investigated for their ability to retain iodide and iodate.

Clay minerals exhibit an anion exchange capacity to some minor extent, because the edges, due to discontinuities in the XY-plane, are positively charged. Anions are adsorbed to these positions and may, especially at higher pH-values, be exchanged by hydroxide ions (Gast, 1977).

The adsorption of iodide on a series of clay minerals has been investigated. Whitehead (1974a) concluded that iodide, in concentrations of 7.88×10^{-7} M and 3.15×10^{-6} M was not adsorbed on kaolinite or montmorillonite. On the other hand, Raja and Babcock (1961) found that minor amounts of iodide could be adsorbed on kaolinite, montmorillonite and bentonite. The presence of dissolved calcium salts decreased the adsorption ability of the two latter clay types.

Rancon (1988) reported low but appreciable retention of iodide on alumina silicates (clay), iron and aluminum ores and on clayey calcareous soils. Much higher retention on certain lead and copper ores were found. A high retention of iodide and iodate was also reported by Strickert et al. (1980) when pure minerals containing copper, lead and iron were investigated. Anderson et al. (1982) concluded that significant adsorptions of iodide on minerals containing Hg, Pb or Ag occur. Haq et al. (1989) demonstrated that Cu_2O, CuO and Cu adsorbed iodide and also the formation of CuI . Strickert et al. (1980) noted that more common geological materials such as granite, basalt and tuff show very little retention of iodide and iodate.

Whitehead (1981) investigated the ability of different soil constituents to reduce the loss of iodine (supplied as iodide) to the air. It was concluded that

mixtures of montmorillonite/sand and kaolinite/sand reduce the loss to 44 and 76%, respectively, compared with pure sand (100%). Apparently, the two clay minerals appear more effective in reducing iodine loss to the air than to the interstitial water. In both cases montmorillonite appears as the more effective clay mineral (pH < 7). Finally, it can be mentioned that Vinogradov (1959) reported illite as the more effective clay type. However, it can probably be concluded that clay minerals are considered to be of lesser importance as soil constituents in the sense of an iodide-adsorbing property.

Sesquioxides, Al_2O_3 and Fe_2O_3 have been reported to influence the retention of iodine in soil (Whitehead, 1973a,b; 1974a,b). Thus, a positive correlation between iodine content and the content of sesquioxides in 23 English soils could be established. Furthermore, it was demonstrated that freshly precipitated Fe_2O_3 and Al_2O_3 could adsorb iodide. A pronounced effect on the adsorbing ability of the sesquioxides towards iodide as a function of pH was noted. The adsorbing ability decreases with increasing pH. Thus, Fe_2O_3 and Al_2O_3 do not adsorb iodide at pH-values above ca. 7 and 8, respectively.

In a review of anion-adsorption, Parfitt (1978) concluded that similarities in the mechanisms for adsorption of different anions apparently exist. Protonation of M-OH groups on the mineral surface leads to positively charged sites and, hence, attraction and adsorption of anions. Maximum anion adsorption takes place at rather low pH-values, close to the pKa-value for the acid $M\text{-}OH_2^+$. Parfitt (1978) suggests that the adsorption of halides at low pH takes place due to electrostatic attraction as

$$M\text{-}OH_2^+ + I^- \longrightarrow M\text{-}OH_2^+\text{-}I^-$$

A wide variety of anions appear to be more susceptible to adsorption than the halides. Thus, in the eventual competition for the relatively small number of sites available, it seems likely that iodide will not be adsorbed to any major extent. On the other hand, this might suggest that the significant adsorption

of iodide on Fe_2O_3 and Al_2O_3 reported by Whitehead (1974a) is a result of the absence of concurrent anions in these experiments.

Finally, it might be mentioned that drying, as long as organic matter is absent, influences the adsorption process only slightly. Hence, it can be concluded that the mineral components of soil probably do not play any predominant role (Benes, 1985) except in the case of rather extreme conditions, where electrostatically controlled reaction mechanisms are dominant. In some cases minerals containing Cu, Pb or Hg may adsorb considerable amounts of iodide, but in most soil and groundwater systems the concentrations of those minerals are so small that they probably can be ignored.

4. THE ORGANIC GEOCHEMISTRY OF IODINE

In 1961 Raja and Babcock studied the effect of various treatments of the soil on amounts of ^{131}I that could be extracted from contaminated samples with various reagents. The treatments included autoclaving, steam bath digestion, digestion with alcohol and hydrogen peroxide treatment. In general untreated soils retained 85-95% of the applied radioiodine while autoclaving remarkably reduced the retention. In addition, it was demonstrated that the organic fraction exhibited the most pronounced ability to retain radioiodine. These results indicated that microorganisms were responsible, but Raja and Babcock themselves rejected this proposal because 80% ethanol, as saturating liquid, which would have killed the microorganisms, did not reduce the retention of iodide. Digestion of the organic matter by hydrogen peroxide reduced the retention of iodide when the saturating liquid was water, but not when it was ethanol.

Whitehead (1973 a) showed too that removing the organic matter with hydrogen peroxide reduced the retention of iodide, and it was also demonstrated that the retention of iodide decreased with depth of the soil sample. For the eight soils examined, the correlation coefficient between the value for sorption at an equilibrium solution concentration at 500 μg I/100 mL and organic carbon content was highly significant ($r = 0.98$).

Whitehead (1975) found that the addition of organic matter to soil markedly reduced the uptake of iodine from soil to plants. The species of iodine used was iodide, iodate and elemental iodine. Quite in concordance with this, Szabová (1976) demonstrated that the presence of increasing amounts of organic material in soil increased the retention of iodine.

Whitehead (1978) found a very poor correlation between organic content and content of iodine in 154 soil samples. This result demonstrated that even if there is a pronounced tendency for organic matter to retain iodine, other factors also play a crucial role in determining the iodine content in soils.

Behrens (1982) investigated the behaviour of iodide under environmental conditions and noted that in surface water iodide was converted to non-iodide. The non-iodide was for a greater part later identified as organic iodine with a molecular weight in the range of 500-10,000. The conversion increased with time, but levelled out after a while. The conversion could be suppressed by heat and when heated to boiling the solution almost lost its conversion ability. On the other hand, the iodide-converting ability could be re-established by grafting the samples with non-sterile water or even house dust.

The reaction needed air and when the solution was filtrated through a 0.2 μm filter both the filtrate and the filter showed conversion ability. These findings proposed that not only microbes but extracellular enzymes aswell are involved in the conversion.

The conversion of iodide to non-iodide seems to be reversible since removing the iodide from the liquid fraction resulted in the release of iodide to an extent that the original ratio of non-iodide to iodide at 80 : 20 was re-established. Addition of sulphite to the water samples resulted in a partial reduction of the non-iodide to iodide.

Addition of d-glucose or hydrogen peroxide increased the iodide-converting activity to a remarkable extent. On the background of the latter results Behrens suggested the involvement of enzymes of the peroxidase group which need hydrogen peroxide to catalyze reactions. The reason that the addition of d-glucose increases the conversion rate could be that the enzyme glucose oxidase if present catalyzes the formation of hydrogen peroxide from glucose.

Behrens (1982) demonstrated also that the ability to convert iodine exists in soil extracts too and that the organically bound iodine which is dissolved to some extent is adsorbed and retained by soil. This is more pronounced in

non-sterile than in sterile soil. It was noted that the conversion ability decreased with depth of the soil sample. Behrens (1982) proposed that the organic iodine was in fact iodinated proteins. In Behrens (1985 and 1986) this proposal about the character of the organic iodine is changed and it is proposed that iodinated humic substances is formed.

It was pointed out by Benes (1985) that the quality of the organic content in soil affected the retention of iodide and that the ability to be subject to iodination was greater for the water soluble organic fraction. These results will partly explain why Whitehead (1978) found a poor correlation between organic and iodine content in soils. This picture is furthermore supported by the observation that a podsolic soil with the highest organic content exhibited the lowest retention ability of iodide (Benes, 1985).

Bors et al. (1984) demonstrated that sterilizing soil by ^{60}Co-radiation lowers the amount of iodide which was retained by the soil compared to unradiated soil samples and concluded that microorganisms were responsible for the retention. Later Bors et al. (1989) demonstrated a general increasing tendency of soil with increasing organic content to retain iodide and that the addition of compost to a soil layer with organic C-deficiency sharply increases the retention.

It was noted by the same authors that addition of anthropogenic complexing agents dibuthylphosphate (DBP), nitrilotriacetic acid (NTA) and ethylen-diamine-tetraacetic acid (EDTA) remobilised the iodine to some extent. Compared with control experiments, the mobilization of the radioiodine was about 2-6 fold but compared with the amount of iodine which was still retained the remobilised fraction was very small.

On the background of the results of Behrens (1982 and 1985), El-Kekli and Johanson (1986) tried to identify the organic iodine containing products formed in soil, by various extraction methods. They found a high molecular fraction that contained iodine with the first water extraction and subsequent

hydroxide extraction. They concluded that the results they had obtained supported the proposal of humic substances as accepter molecules for iodine.

Behrens (1985) confirmed the results of Behrens (1982) by batch type experiments and soil column experiments. By column experiments it was demc ıstrated that 77.75% of the radioiodine was fixed in the upper 5 mm and that the fixed amount of iodine decreased with depth.

Behrens (1986) found that "iodide converting" reactions take place in water from deep aquifers, but he concluded that in spite of careful handling it cannot be excluded that secondary microbial growth and subsequent formation of "iodide converting ability" is responsible for the observation.

Dertinger et al. (1986) obtained results which are almost identical to those found by other authors. They noted for instance that iodide was converted to non-iodide which then was retained by soil. This result can be compared with the results obtained by Behrens (1982) who noted that dissolved iodine containing compounds which were formed in surface water to a certain extent was retained by soil.

Asplund et al. (in press) reported that the enzyme chloroperoxidase from the fungus *Caldariomyces fumago* is able to catalyze the chlorination of fulvic acid and that soil enzymes are able to convert monochloro-dimedon to dichloro-dimedon The latter reaction is normally used as an assay for chloroperoxidase activity. It seems reasonable that both iodine and chlorine and possibly bromine are subject to enzymatically controlled incorporation into humic substances under natural conditions in soil and groundwater systems.

In marine sediments an alternative mechanism for iodine fixation is suggested. In order to elucidate the organic fixation of iodine by humic material in marine sediments, Francois (1987) treated humic material with iodate and resorcinol at pH 2.5 - 3. Due to the subsequent observation of

iodinated resorcinol he concluded that humic material reduces IO_3^- to an iodinating species, I_2 or HOI which then iodinate humic substances and aromatic molecules as resorcinol. However, if iodide is also present, iodate and iodide will form elemental iodine at the applied pH.

The possibility of peroxidase catalyzed iodination of humic substances in the terrestrial environment has been discussed by Christiansen and Carlsen (1989). Experimental results confirm that lactoperoxidase catalyzes the iodination of humic acid in the presence of iodide and hydrogen peroxide (Christiansen and Carlsen, 1990 in press). In addition it has been demonstrated by Christiansen and Carlsen (subm.) that other peroxidases such as horseradish peroxidase and chloroperoxidase catalyze the reaction as well.

There seems to be remarkable agreement between the results obtained by different authors. Iodine is without doubt fixed to the organic fraction in soil and in the following chapters the possible enzymatically controlled iodination of humic substances in the terrestrial environment will be further elucidated by model experiments applying commercially available enzymes and humic acid.

The following questions form the basis for the experimental work:

Do peroxidases catalyze the iodination of humic substances?
If they do, then to what extent?
Do other reactions (non enzymatic) incorporate iodine into humic substances?
What kind of sites in humic substances are iodinated?
Is the reaction reversible or partly reversible?
Do isotope exchanges occur?
Do these enzymes exist in soil systems?

5. INTRODUCTION TO THE EXPERIMENTAL WORK

The experimental work was planned to consist of three different groups of experiments.

In the first group phenol was selected as a simple model compound for humic substances. It was the aim of this part of the work to elucidate the enzymatically controlled iodination of a simple organic molecule and develop an iodination method which could act as a basis for planning more realistic experiments involving humic acid. In addition, it was expected that some hints about the reaction mechanism on the molecular level would show up. Experiments using elemental iodine as iodinating species were planned to serve as reference. Cooksey et al. (1985) investigated eight aromatic compounds for "antithyroid effect"; all were well-established breakdown products from humic acid. It was decided to include these compounds in the investigations.

The second group of experiments involved humic acid as acceptor molecules for elemental iodine and the iodinating species formed from iodide, hydrogen peroxide and lactoperoxidase. The concentrations of the single reactants were selected in ranges which could be expected to be realistic under environmental conditions. The major part of the experiments have been carried out using lactoperoxidase in accordance with the suggestion of Behrens (1982). However, it was more realistic to suppose that enzymes produced by plants or fungi occur in soil systems; for that reason, experiments were also carried out using horseradish peroxidase and chloroperoxidase, the latter formed from the fungus *Caldariomyces fumago.*

The third group of experiments involved "naturally occurring iodinating ability", extracted from Swedish soil and believed to be due to peroxidase activity.

To analyze the products formed in the experiments, a HPLC system equiped

with a UV-detector and a radioactivity detector was used. The products obtained from iodination of phenol and phenol availabel in one of the other aromatic compounds were analyzed applying a 250 x 4.6 mm Nucleosil C8 (5μ or 10μ) column. Eluent: MeOH/H_2O v/v 50/50 and 62/38, respectively. The flow rates were 1 and 0.6 mL/min, respectively. The products formed from enzymatically controlled iodination and from iodination by elemental iodine of all the aromatic compounds were analyzed by means of field ionization/field desorption (FI/FD) mass spectrometry. The products formed from enzymatically controlled transformation of mono-iodophenols were analyzed by means of mass spectrometry (FI/FD). The products formed from bromination and iodination of phenol in a large excess of phenol was analyzed by gas chromatography in which a 30 m x 0.52 mm capillary DB-1 column (150°C isotherm./FID) was applied. The product formed from iodination of phenol by the chloramine-T method was analyzed by means of mass spectrometry (FI/FD) and HPLC as above. The products formed from transformation of mono-iodophenols by chloramine-T were analyzed by means of mass spectrometry (FI/FD).

The qualitative analysis of enzymatically controlled iodination of humic acid was carried out by means of HPLC. The column applied was a 250 x 4.6 mm Ultrahydrogel™; the eluent was 0.1 M sodium acetate adjusted to pH 9.6, and the flow was 0.6 mL/min. The quantitative analysis was carried out by acid precipitation of the humic acid, centrifugation, and subsequent removal of a known volume of the supernatant to another tube. The two tubes were then subject to gamma counting until 100 000 counts were registered. The number of counts per min served as an expression for the relative amount of iodine in the two vessels, and the amount of iodine fixed in the humic acid was then calculated based on the total volume and the volume of supernatant transferred to the other vessel.

The analysis of the product formed from "naturally occurring iodination ability" was carried out by means of HPLC as in the case of enzymatically controlled iodination of humic acid above.

Experimental details can be found in the publications "Iodination of phenol", "Iodinated humic acids" and "Enzymatically controlled iodination reactions in the terrestrial environment" in Appendices 4, 5 and 6. Material and methods for experiments unavailable elsewhere can be found in Appendix 1.

6. IODINATION OF MODEL COMPOUNDS

6.1 Iodination of phenol

In a recent study phenol was iodinated by elemental iodine (I_2) or by iodide in the presence of lactoperoxidase and hydrogen peroxide (Christiansen, Feldthus and Carlsen in press). It was demonstrated by means of MS that compounds with masses corresponding to mono-, di- and tri-iodophenols were formed. Six aromatic compounds were identified in the crude product; phenol, 2-iodophenol, 4-iodo-phenol and 2,4,6-tri-iodophenol were identified by means of HPLC and authentic compounds; 2,6- and 2,4-di-iodophenol were separated by means of preparative HPLC and the structures confirmed by ^{1}H-NMR and ^{13}C-NMR. The structures of the compounds found in the product are given in Fig. 3.

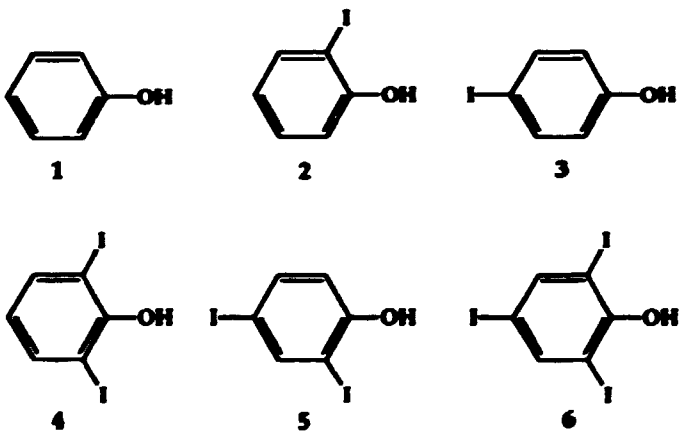


Fig. 3. The structure of phenol and the iodo-phenols formed when phenol is iodinated by elemental iodine.

When phenol was iodinated by iodide (spiked with ^{131}I) in the presence of hydrogen peroxide and lactoperoxidase 5, iodine-containing compounds were obtained; all exhibited the same retention times as the identified compounds formed using elemental iodine (Fig. 4).

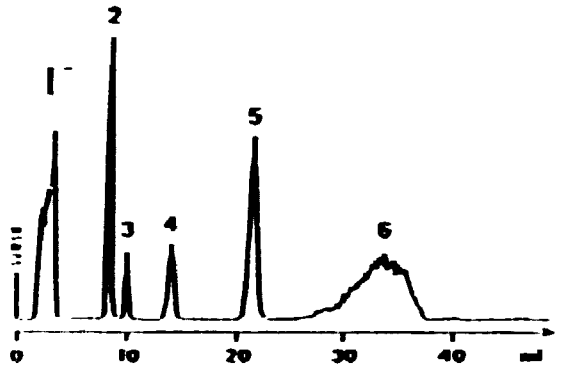


Fig. 4. HPLC trace of the product from enzymatically catalyzed iodination of phenol using ^{131}I; the radioactivity signal is shown.

To confirm that the five compounds were in fact iodo-phenols, the experiment was subsequent carried out using phenol spiked with ^{14}C-phenol and stable iodide. Six ^{14}C-containing compounds were formed. The retention times confirmed that the product consisted of phenol, 2-iodophenol, 4-iodophenol, 2,6-di-iodophenol, 2,4-di-iodophenol and 2,4,6-tri-iodophenol (Fig. 5).

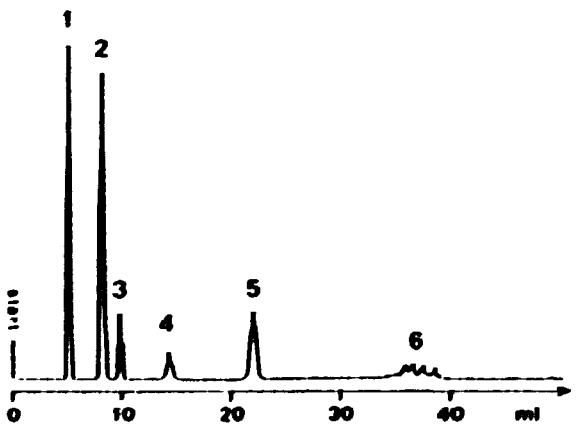


Fig. 5. HPLC trace of the product from enzymatically catalyzed iodination of phenol using ^{14}C phenol; the radioactivity signal is shown.

When the ratio of the initial concentration of phenol to that of iodide was changed, the product distribution, not unexpectedly changed too. With increasing phenol/iodide ratio the product moved towards increasing content of mono-iodophenols, and when the ratio decreased the relative amount of poly-iodophenols increased (Fig.6).

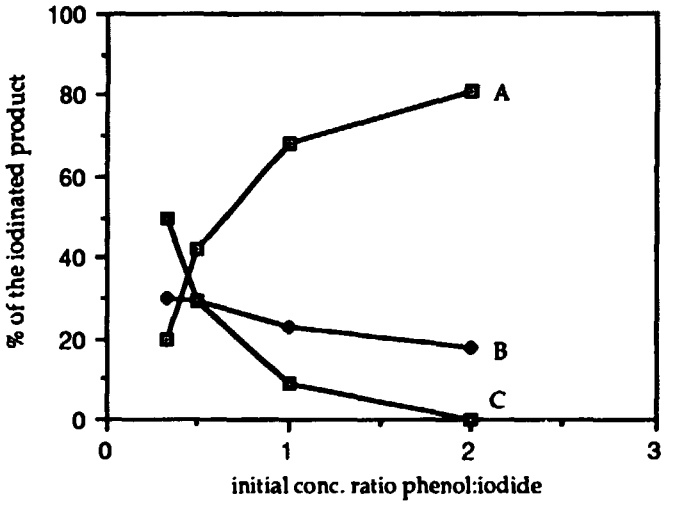


Fig. 6. The distribution of the iodinated product on A, mono-; B, di- and C, tri-iodinated phenols as a function of the initial concentration ratio (phenol : iodide) (the conc. of iodide was $1x10^{-4}$ M).

The enzyme-catalyzed iodination of phenol forming mono-, di- and tri-iodinated phenols can obviously be formulated as a series of consecutive steps from phenol to tri-iodophenol via the formation of mono- and di-iodophenols (Fig. 7).

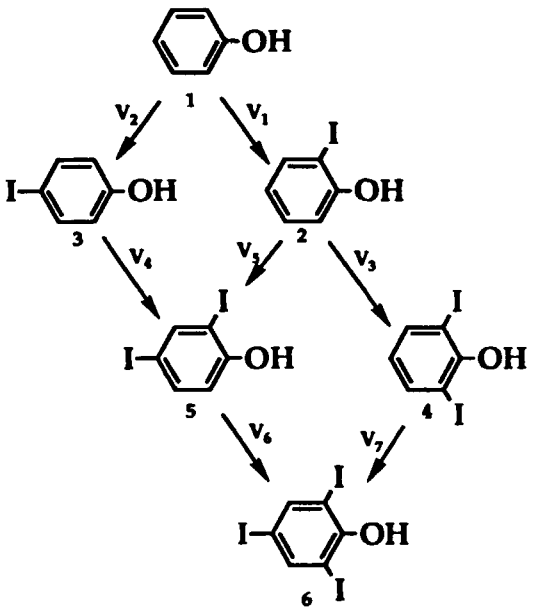


Fig. 7. Scheme of the series of consecutive reaction steps from phenol to 2,4,6-triiodophenol.

In order to determine the relative rates of the "competitive" iodination reactions, different combinations of phenol and iodophenols were iodinated enzymatically, the phenols being in large excess. Thus, only the first iodo derivatives from the parent molecules were formed. Using this method it is possible to measure only the relative rates, and because the results are a little scattered only the relative order of the rates can be obtained. The relative order of the reaction rates is determined on the basis of the product distribution and is listed in Table 1.

Table 1. Relative order of iodination rates based on the product destribution.

Reactants	Rate relation
Phenol,	$V_1 > V_2$
2-iodophenol	$V_3 > V_5$
Phenol, 2-iodophenol	$V_3 \geq V_1 + V_2$
	$V_5 \sim V_1 + V_2$
Phenol, 4-iodophenol	$V_4 \sim V_2$
Phenol, 2,4-diiodophenol	$V_7 >> V_1$
Phenol, 2,6-diiodophenol	$V_6 << V_1$

As can be seen from Table 1, the ortho-position in phenoi relative to the para-position is favoured for iodination. In further iodination of 2-iodophenol, the second ortho-position is only slightly favoured compared with the para-position. When the para-position is occupied by iodine, further iodination is much less pronounced. When the two ortho-positions are occupied by iodine, the para-position is strongly favoured for further iodination relative to the formation of mono-iodophenol from phenol.

It is interesting to note that only the iodine in the para-position is subject to enzymatically catalyzed isotope exchange. Also of interest is that enzymatically controlled iodination of phenol in excess of hydrogen peroxide (compared to the conc. of iodide) causes the formation of 2-iodophenol dimers and that 4-iodophenol is not subject to dimerisation. The 2-iodophenols are probably linked through the other ortho-carbons. Danner et al. (1973) demonstrated the enzymatically catalyzed dimerisation of phenol through the ortho-carbons.

It is possible that HOI is the iodinatig species when aqueous solutions of

phenol are iodinated by elemental iodine. When phenol (3.3 x 10^{-3} mole/L) was iodinated at pH 5 by elemental iodine (3.3 x 10^{-4} mole/L) , 6 - 7 % of the mono-iodophenol formed was 4-iodophenol and 93-94% was 2-iodophenol. When elemental iodine was treated with equivalent amounts of NaOH the solution turned colourless suggesting that HOI is formed. After adjusting pH to 5 by acetatebuffer (the solution was still colourless), phenol was added. The product constituted of 2-iodophenol and 4-iodophenol in the same ratio as above. This suggests that the same iodinating species probably HOI acts in the two cases.

However, bromination of phenol on applying elemental bromine leads to the formation of only 23% of 2-bromophenol, in agrement with recent results reported by Tee et al. (1989), who found a preference for para substitution at pH 4. In Table 2 some ortho/para ratios for halogenation of phenol in aqueous solutions are listed.

Tabel 2. Ortho/para ratios of the products formed, halogenating phenol

Reaction	pH	ortho/para	
Phenol, Bromine	pH 5	0.30	(Christiansen et al. in press)
Phenol, Iodine	pH 5	13.3	
Phenol, NaOCl	pH 4	0.64	(Ogata et al. 1989)
Phenol, NaOCl	pH 7	1.8	
Phenol, NaOCl	pH 8.8	2.8	
Phenol, NaOCl	pH 10	4.3	

From Table 2 it can be seen that at pH 5 the ortho-position is strongly favoured for iodination, while in the case of bromination the para-position is favoured. At alkaline pH, Tee et al. (1989) suggested that the ortho/para ratio changed towards increasing values when brominating phenol. However, they used equal amounts of phenol and bromine and hence polybrominated

phenol was formed. This may distort the ortho/para ratio by possible preferential consumption of ortho-bromophenol (Tee et al., 1989).

After making theoretical calculations, Ogata et al. (1984) suggested that the para-position in phenol, in the phenoxide ion and in anisole should be favoured for electrophilic substitution compared with the ortho-position. However, they suggested that some of the calculations support a mechanism which involves the formation of PhOCl; this may rearrange as ortho-chlorophenol. The mechanism is further supported by the increase of the ortho/para ratio increases with increasing pH (Table 2) and that chlorination of anisole leads to the expected ortho/para ratio based on the theoretical calculations. A comparable mechanism may be involved in the iodination of phenol.

Chloramine -T mediated iodination of phenol in acetatebuffer (pH 5) leads to an ortho/para ratio between 0.5 and 1. However, the iodide/phenol ratio was 1 and the formation of a considerable amount of di-iodophenol was observed. It can be noted that only 2.4-di-iodophenol and not 2.6-di-iodophenol was observed which suggests that the di-iodophenol was formed from 4-iodophenol. Kometani et al. (1985) found 96% formation of 4-iodophenol by chloramine-T mediated iodination of phenol in dimethylformamide and dimethylsulfoxide. These findings suggest that chloramine-T mediated iodination involves a mechanism other than iodination by elemental iodine.

When treating 2-iodophenol and 4-iodophenol with chloramine-T in acetatebuffer (pH 5), 2-iodophenol is not detectable by means of MS after one hour, but 4-iodophenol seems to be unaffected. These findings may distort the results because selective transformation of 2-iodophenol to some unknown compounds will decrease the ortho/para ratio. It can also be noted that chloramine-T mediated iodination of phenol in acatatebuffer leads to the formation of unknown iodine-containing compounds which was difficult to elute from the HPLC column. By means of MS no dimers of iodophenol were

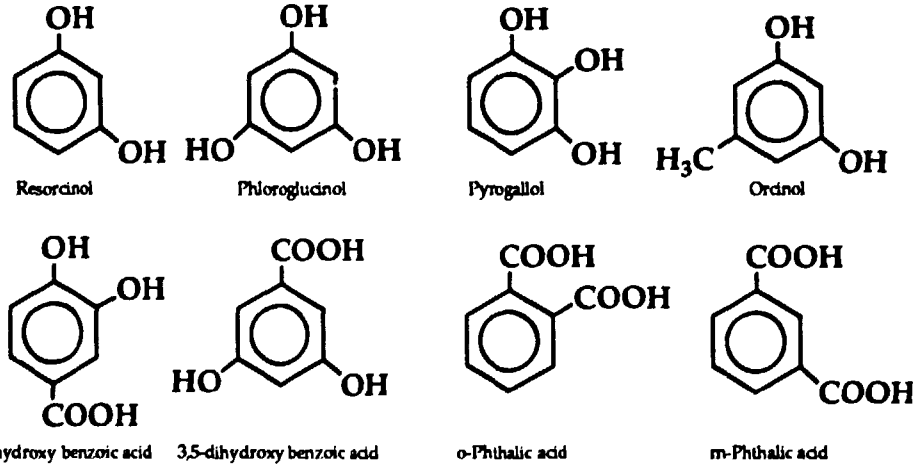
found, contrary to the enzymatically controlled iodination of phenol in excess of hydrogen peroxide where dimers of 2-iodophenol were produced.

Obviously, the halogenation of phenol is not so simple as could be expected, and further elucidation of the interaction of different halogenating species with phenol is necessary if the mechanisms involved are to be described in detail.

6.2. Iodination of "antithyroid" compounds

Cooksey (1985) described how different aromatic compounds caused a diminished incorporation of ^{125}I in thin slices of hog thyroid gland and disturbed the function of thyroid glands in rats. It was concluded that the function of the thyroid peroxidase, responsible for the iodination of the thyroid hormone, was inhibited by these compounds.

As a part of our current investigations of enzymatically controlled iodination of humic substances in soil, the eight aromatic compounds investigated by Cooksey (1985), i.e. resorcinol, phloroglucinol, pyrogallol, orcinol, 3,5-dihydroxy benzoic acid, 3,4-dihydroxy benzoic acid, ortho-phthalic acid and meta-phthalic acid caught our attention, owing as these compounds are all well-established breakdown products from humic acids.



The observed disturbed function of the thyroid gland can be ascribed to inhibition of the enzyme thyroid peroxidase. Thus, the apparent inhibition could a priori be due either to competitive iodination reactions involving the so-called "antithyroid" aromatic compounds at the expense of the thyroid hormone or to structural blocking of the enzyme activity.

To elucidate whether the above-mentioned aromatic "antithyroid" compounds could possibly influence the activity of the lactoperoxidase, the enzymatically controlled iodination of phenol was carried out in the presence of the aromatic compounds noted above. The results demonstrated a trend that was the same as the one noted by Cooksey (1985). Resorcinol, phloroglucinol, orcinol, and 3,5-dihydroxy benzoic acid inhibited the iodination of phenol completely while 3,4-dihydroxy benzoic acid, ortho-phthalic acid and meta-phthalic acid did not (in concentrations of 10^{-4} M). In addition, it should be noted that orcinol was iodinated to a minor extent. Pyrogallol was not investigated in the present study as it immediately forms purpurogalin with hydrogen peroxide and lactoperoxidase.

It was demonstrated by application of mass spectrometric analysis that elemental iodine is able to iodinate resorcinol, phloroglucinol, orcinol and 3,5-dihydroxy benzoic acid and to a very minor extent (trace) 3,4-dihydroxy benzoic acid, but not ortho-phthalic and meta-phthalic acids. Thus the above lack of enzymatically controlled iodination of the "antithyroid" compounds cannot be explained by an absence of reactivity towards iodination in general. On this background, it seems reasonable to conclude that the fact that the "antithyroid" compounds in general were not subject to enzymatically controlled iodination reflects a blocking of the enzyme by these compounds. However, the mechanism is not quite obvious as some iodination of orcinol seems to prevail. On the other hand, a support for competitive iodination can be mentioned, as Fawcett and Kirkwood (1953) found iodoresorcinol when treating thin slices of rat thyroid with iodide and resorcinol.

Based on the above, the product mixtures obtained from the aromatic

compounds treated with iodide, hydrogen peroxide and lactoperoxidase in acetatebuffer pH 5 were analyzed using MS to elucidate whether iodinated species which were not detectable using HPLC were formed. It was demonstrated that only mono iodoorcinol, mono-,di- and tri-iodophenol were formed and to a minor degree 4-iodo-3,5-dihydroxy benzoic acid, the latter confirmed by means of HPLC.The results are summarized in Table 3.

Tabel 3. Iodination of "antithyroid compounds" by elemental iodine or catalyzed by lactoperoxidase and enzymatically controlled iodination of phenol in the presence of one of the "antithyroid compounds".

Antithyroid compounds.	I_2	Enzymatic. iodination	Inhibition of phenol iodin.	Antithyroid-effect x)
Resorcinol	m,d & t	-	+	+
Phloroglu.	m,d & t	-	+	+
Orcinol	m & d	m	+	+
3,4-dihydroxy benzoic acid	m (trace)	-	+	+
3,5-dihydroxy benzoic acid	m	m (trace)	+	+
o-phthalic acid	-	-	-	-
m-phthalic acid	-	-	-	-

m = monoiodinated, d = diiodinated and t = triiodinated

x) Cooksey et al. (1985)

7. IODINATION OF HUMIC ACID

7.1 Verification of iodination

To confirm that enzymatically controlled iodination of humic acid is actually able to take place, humic acid (Aldrich) was allowed to react with iodide, hydrogen peroxide and lactoperoxidase for 20 min at room temperature. It was demonstrated that significantly higher concentrations of enzymes (5-10 fold) were necessary to get a comparable amount of iodide incorporated into humic acid in the time range of 20 min as into phenol in the time range of 10 min, even though the concentration of humic acid was 100 mg/L and that of phenol was only 10 mg/L. But it was demonstrated, still in rather low concentrations of the reactants, that the incorporation in the presence of hydrogen peroxide does occur under laboratory conditions as visualized in Fig. 8.

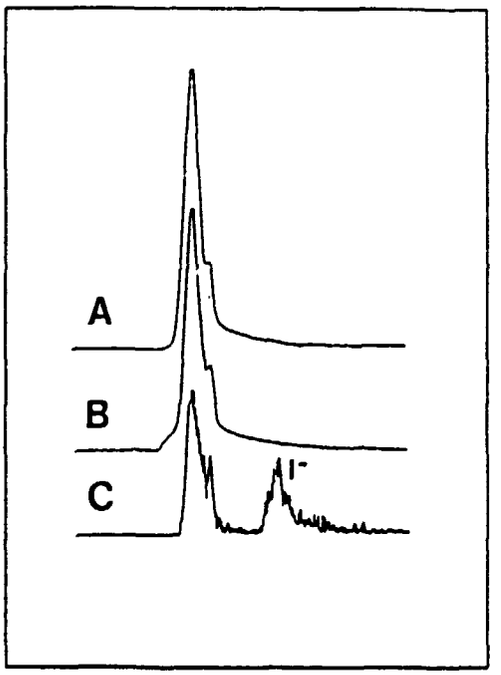


Fig. 8. Chromatographic trace of A: humic acid prior to iodination , B: humic acid after iodination (UV detection), C: humic acid after iodination (^{131}I detection). Initial concentrations: humic acid ; 0.1 g/L; iodide, 3×10^{-5} M; hydrogen peroxide, 2×10^{-4} M; enzyme, 10 μg/mL. Reaction time 20 min.

Control experiments demonstrated that no incorporation was detectable in the time range of 20 min without applying both enzyme and hydrogen peroxide. It can be concluded that iodide is incorporated into humic acid through the action of lactoperoxidase.

It is possible that the incorporation of iodide into macro molecules of humic substances would proceed via the incorporation into smaller molecules followed by cross coupling reactions forming macro molecules. Sakar et al. (1988) demonstrated the peroxidase-catalyzed incorporation of 2,4-dichlorophenol into stream fulvic acid. In the present case this possibility may be rejected because a uniform distribution of the iodide in the humic acid weight fractions propose no preferences. The UV trace of the humic acid does not change during the iodination reaction supporting the view that no pronounced change of the original weight fraction distribution takes place. The uniform distribution of iodide suggests that this humic acid is built up of rather equal units or at least that the sites which can be subject to iodination are rather equally distributed in a'l weight fractions. From these results, it seems obvious that a direct iodination of dissolved humic macro molecules does occur.

In addition it was demonstrated that elemental iodine (spiked with ^{131}I), formed from iodate and iodide, also was able to iodinate humic acid and that the iodine was uniformly distributed in the humic acid fractions. However applying this method a lower yield was obtained possibly due incomplete formation of elemental iodine.

7.2. Influence of the enzyme concentration

Morrison and Bayse (1970) demonstrated a linear correlation between the iodination rate of tyrosine and enzyme concentration. To confirm that the iodination rate of humic acid also increased with an increasing enzyme concentration, the concentration of enzyme was varied from 2-20 µg/mL and the amount of iodine which was incorporated into the humic acid in 10 min

was plotted as a function of the enzyme concentration.

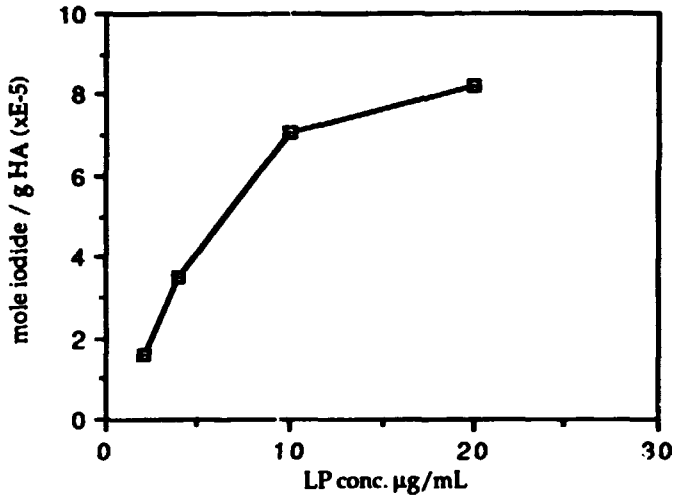


Fig. 9. Iodine incorporation as a function of enzyme concentration (initial concentrations: iodide, 3 x 10^{-5} mole/L; hydrogen peroxide, 2 x 10^{-4} mole/L; humic acid, 0.1 g/L).

We did not obtain a linear correlation. The amount of incorporated iodine in 10 min reflects a decreasing rate because the concentrations of iodide, hydrogen peroxide and available sites in the humic acid decrease as the reaction proceeds. This results in a greater reduction of the reaction rate in the case of higher enzyme concentrations because the concentrations of iodide, hydrogen peroxide and available sites decrease faster as the reaction proceed. If one assumes that the curve start at 0.0, the first part of the curve between 0.0 and the two first points is linear. This is quite in accordance with the explanation given above because a low reaction rate due to low enzyme concentration will consume only minor amounts of the reactants involved during the 10 min of reaction time. It can be noted that less than 15% of the iodide is incorporated in 10 min when the enzyme concentration is 4 µg/mL, corresponding to the second point in the curve.

7.3. Influence of the humic acid concentration

If iodide were present in excess it would be expected that the amount of incorporated iodine would increase almost linearly with the humic acid concentration. When the humic acid concentration is varied from 0.05 g/L to 0.4 g/L, the incorporation increases from 8.6 x 10^{-6} to only 3.5 x 10^{-5} mole/L. This means that when the humic acid concentration increases 8 fold the incorporation increases only 4 fold. When the concentration of humic acid is increased to 1 g/L the incorporation increased no further.

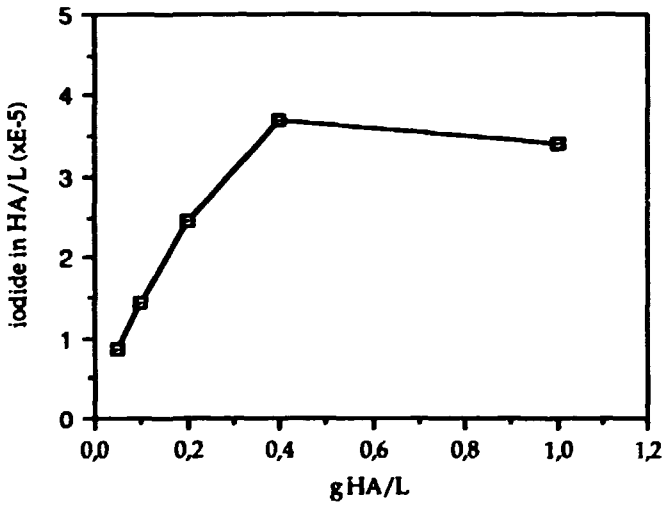


Fig. 10. Iodine incorporation as a function of humic acid concentration (initial iodide concentration, 1 x 10^{-4} M).

It is quite obvious that neither a deficiency of available sites nor the decrease in iodide concentration as the reaction proceeds can explain the results. One plausible explanation is that under the present conditions the concentration of humic acid (above 0.4 g/L) is so high that it is not a part of the rate determining-step of the reaction.

Another explanation could be that the humic acid inhibits the function of the enzyme and that an increased concentration of humic acid causes an increased inhibition of the enzyme and a subsequent increased reduction of

the incorporation. There seems to be a pronounced support for this suggestion in other publications. For instance, the association of humic acid with enzymes and subsequent reduction of the activity is generally believed to take place in soil systems (Burns in Burns 1978 and Tate 1987). It was demonstrated by Serban and Nissenbaum (1986) that humic acid forms stable associations with catalase and peroxidase and that these stable complexes still retained a conciderable portion of the original activity afterwards.

To clarify, if the humic acid reduced the iodination activity as well, lactoperoxidase have been incubated with humic acid for one hour prior to the addition of iodide and hydrogen peroxide.

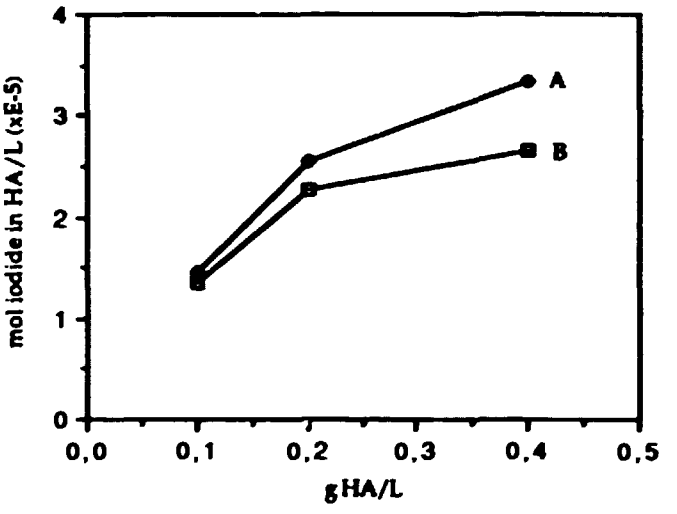


Fig. 11. Iodine incorporation as a function of humic acid concentration. Curve A corresponds to the incorporation when the enzyme was added last and curve B corresponds to the incorporation when the enzyme and humic acid have been in contact 1 hour before initiation of the reaction.

As can be seen from the curves, the incorporated amount of iodine is reduced when the enzyme has been in contact with humic acid before initiation of the reaction. An increased reduction is observed when the humic acid concentration increases stepwise from 0.1 to 0.4 g/L. The reductions are 7%, 11% and 21%, respectively. It could be claimed that competitive inhibition

could be an alternative mechanism, but if humic acid were to act as substrate instead of iodide, precontact would not affect the reaction rate before hydrogen peroxide were added. The results clearly demonstrate that precontact between humic acid and lactoperoxidase causes a reduction of the incorporation of the iodine. It may be concluded that some kind of physical contact, for instance complexation, affecting the overall reaction rate must be the explanation.

7.4. Influence of the iodide concentration

If the reaction time were kept constant and the iodide concentration increased, one would expect the incorporation of iodine into humic acid to increase until the humic acid were saturated with iodine or the maximum turnover number for the appropriate enzyme is reached with the consequence that the iodide concentration would no longer be the limiting factor.

In order to elucidate the influence of the iodide concentration three different series of experiments were carried out. The iodide concentration was varied from 5×10^{-6} to 2×10^{-4} mole/L and the reaction time in the first series was 10 min, the second 4 hours and the third 20 hours. In Fig. 12 the amount of incorporated iodine is plotted as a function of the initial iodide concentration.

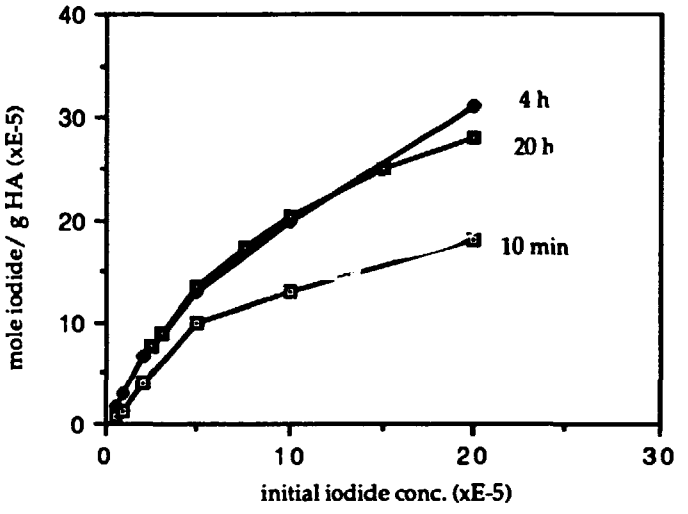


Fig. 12. Iodine incorporation as a function of initial iodide concentration (initial concentrations: hydrogen peroxide, 2 x 10^{-4} mole/L; enzyme, 10 μg/mL; humic acid, 0.1 g/L). Reaction time: 10 min, 4 h and 20 h.

As was expected, the amount of incorporated iodine increases with an increasing initial iodide concentration. The curves representing reaction times at 10 and 4 hours are quite different, but the one representing a reaction time at 20 hours is almost identical to the 4-hour curve. This result indicate that the reaction under the given conditions does not proceed for a period longer than 4 hours or until an equilibrium state is reached. If the percentage of the initial iodide concentration which is incorporated into the humic acid is plotted versus the initial concentration of iodide, a rather surprising picture is obtained.

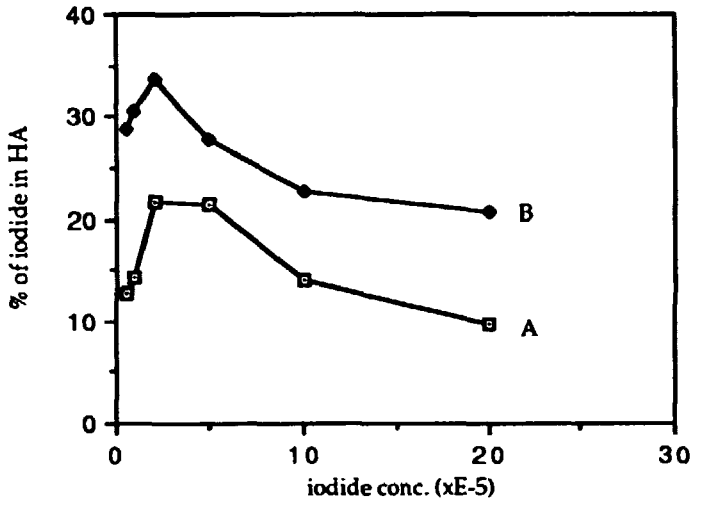


Fig. 13. Percentage of iodine incorporated as a function of initial iodide concentration (initial concentrations as in Fig. 12.) Reaction time; A, 10 min and B, 4 h.

A maximum appears corresponding to an initial iodide concentration of 2.5 x 10^{-5} m/L. One would expect a decreasing percentage to be incorporated with an increasing iodide concentration. Due to the presence of discrepancies from the expected tendency in the diluted iodide concentrations after 4 hours of reaction time, a reasonable explanation can be that competitive reactions of some kind go on in the reaction mixture causing a deficiency of hydrogen peroxide or enzyme. The high excess of hydrogen peroxide relative to iodide will probably cause a breakdown of the enzyme in agreement with the results of Jenzer et al. (1986) who found a good correlation between the recovery of lactoperoxidase and the ratio of iodide to hydrogen peroxide. This finding suggests that the presence of iodide protects the enzyme from inactivation caused by hydrogen peroxide (for an alternative explanation see below).

7.5. Influence of the hydrogen peroxide concentration

To elucidate the extent to which the concentrations of hydrogen peroxide affect the the incorporation rate of iodine into humic acid, a series of

experiments were carried out with identical initial iodide concentrations and varying hydrogen peroxide concentrations.

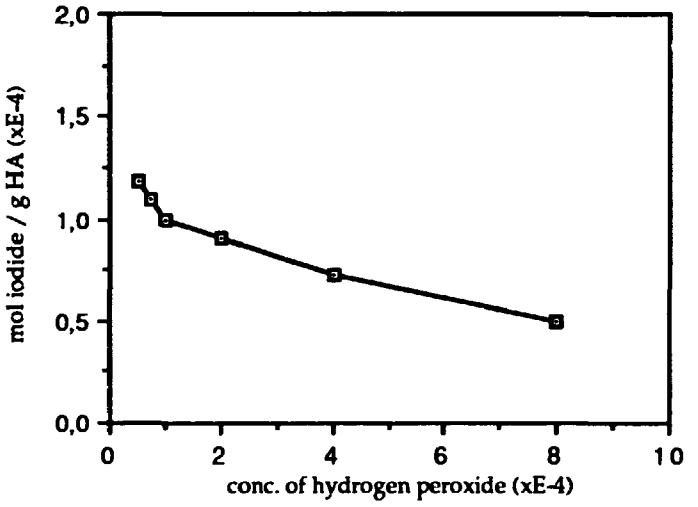


Fig. 14. Iodine incorporation as a function of hydrogen peroxide concentration (Initial concentrations: iodide, 5 x 10^{-5} mole/L; enzyme, 10 µg/mL, humic acid, 0.1 g/L). Reaction time, 10 min.

In Fig. 14 it is clearly demonstrated that the incorporation of iodine into humic acid decreases with increasing concentration of hydrogen peroxide a fact which immediately supports the proposal that peroxidases is inhibited by hydrogen peroxide. However, another explanation can be formulated: Niedleman and Geigert (1986)(and ref. in here) reported that the pH optimum of some haloperoxidases including lactoperoxidase changes with the ratio of iodide to hydrogen peroxide expressed in the equation:

$$\text{optimal pH} = ß + \text{Log}\,[I]/[H_2O_2]$$

where ß = optimal pH when $[I] = [H_2O_2]$

If the optimal pH changes with the ratio of iodide to hydrogen peroxide, the amount of incorporated iodine into the humic acid will decrease when the ratio differs from one. This could obviously explain some of the trends in

our results.

7.6. Influence of the reaction time.

To confirm the conclusion that the incorporation of iodine into humic acid does not proceed for more than 4 hours, the influence of the reaction time was studied in detail. The reaction time was varied from 1 min to 24 hours and the incorporation determined.

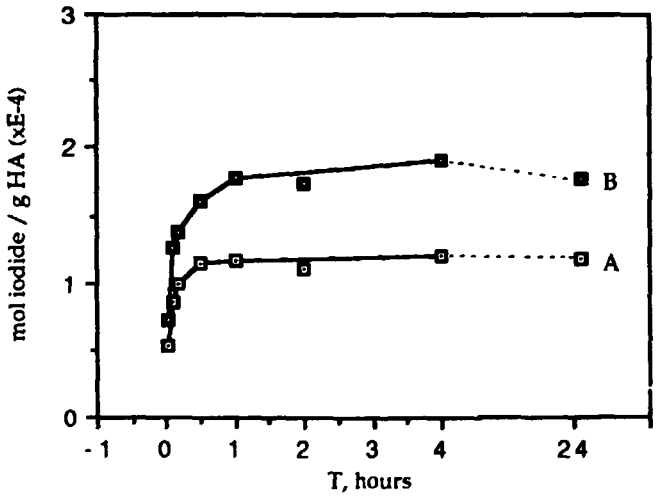


Fig. 15. Incorporation of iodine into humic acid as a function of reaction time for initial iodide concentrations equal to A: 5 x 10^{-5} mole/L and B: 10^{-4} mole/L.

Two initial iodide concentrations were used, and the results clearly demonstrate that incorporation no longer takes place after 4 hours. It can be noted that the two curves level out corresponding to two different amounts of incorporated iodine. The optimal pH can be expected to be different in the two experiments. Based on the expression on page 47 the difference in the initial optimal pH can be calculated as 0.3 pH unit. It does not seem reasonable that a slight difference in the reaction rates will cause a parallel levelling out of the two curves, and a kind of equilibrium state could explain the results as well or even better.

7.7. Equilibrium / reversability.

Based on the following two facts, it appears reasonable to formulate the iodination of humic acid by iodide in the presence of hydrogen peroxide and lactoperoxidase as an equilibrium reaction:

1) Enzymatically controlled iodination of phenol led to 100% consumption of iodide, whereas no iodination of humic acid caused more than 35% consumption of the iodine.

2) Using two different concentrations of iodide (5×10^{-5} and 1×10^{-4}) enzymatically controlled iodination caused the incorporation of different amounts of iodine into the humic acid even after 20 hours of reaction time

In order to describe the potential equilibrium in the system consisting of humic acid, iodide, hydrogen peroxide and lactoperoxidase based on a rather poor knowledge of the mechanism and non at all of the number of sites in the humic acid which are able to be subject to iodination, it was necessary to develop an equation that would produce values for the equilibrium constant and/or the number of sites. The simplest equation that could satisfy these demands took into account only the concentration of the iodinating species ["I"] (assumed equal to the concentration of iodide), the concentration of sites available for iodination [S] and the concentration of iodinated sites [SI].

S + "I" -> SI

This equilibrium gives rise to the following expression

$[SI]/[S]["I"] = K_{eq}$

Which can be rewritten into (details are given in Appendix 3)

$$[I]_o/(1+Q) = q\,[HA]/Q - 1/K_{eq}$$

where

$[I]_o$ = initial iodide concentration

$Q = [SI]/["I"]$

[HA] = concentration of humic acid in g/L

q = number of sites in moles/g HA

K_{eq} = equilibrium constant

To develop the expression two more assumptions must be accepted.

$$[I]_o = [SI] + ["I"]$$

$$S_o = [SI] + [S]$$

where

S_o = q [HA] which is the number of sites in a given amount of humic acid.

S = the number of sites which is not iodinated.

To get the results which could be put into the expression humic acid in concentrations of 0.1 g/L was iodinated using different initial concentrations of iodide. The reaction times were 20 hours for each based on the assumption that equilibrium was reached after that period according to the results above. In Fig. 16 $[I]_o/(1+Q)$ is plotted as a function of [HA]/Q. According to the expression, the slope gives the number of sites in moles per gram of humic acid, and from the intercept of the y-axes the equilibrium constant K_{eq} can be calculated.

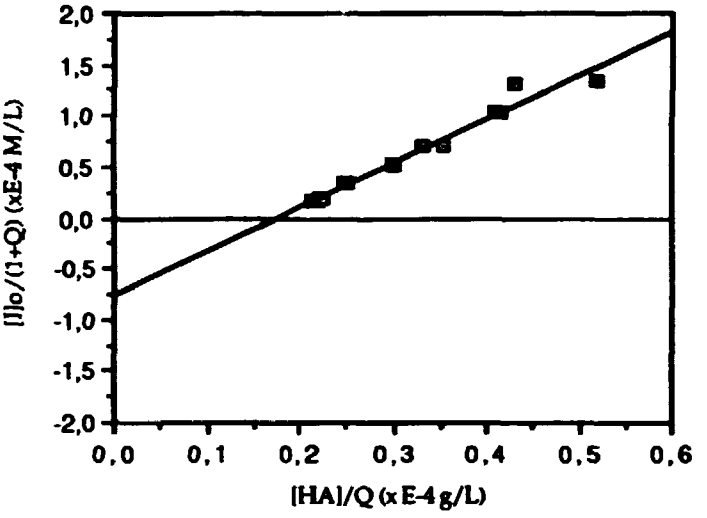


Fig. 16. Plot of initial iodide concentration/(1+Q) as a function of [HA]/Q. The initial iodide concentration is varied from 2 x 10^{-5} to 2 x 10^{-4} mole/L. The humic acid [HA] concentration is 0.1 g/L; enzyme, 10µg/mL; hydrogen peroxide, 2 x 10^{-4} mole/L.

It is immediately seen that a linear correlation between $[I]_o/(1+Q)$ and [HA]/Q was obtained. The correlation coefficient is about 0.99 which strongly suggests that the simple equilibrium model proposed above is applicable to describe the enzymatically controlled iodination of humic acid. Based on a least square procedure, the number of sites avai.able for iodination as well as the equilibrium constant were calculated giving q = 4.28 ± 0.22 x 10^{-4} mole sites/g HA and K_{eq} = 1.32 ± 0.17 x 10^{4} L/m.

Niedleman and Geigert (1986) noted as one of the characteristics of halo-peroxidase catalyzed reactions that they are irreversible. This seems to be in contradiction to the proposed equilibrium model of enzymatically controlled iodination of humic substances and also in contradiction to the results obtained by Behrens (1982, 1985 and 1986).

To confirm that the reverse reaction SI -> S + I does proceed, small portions of $^{131}I^-$ labelled iodinated humic acid were isolated and dissolved in acetate

buffer pH 5. In order to study the possible deiodination process and elucidate the factors affecting the release of iodine from humic acid lactoperoxidase, stable iodide, hydrogen peroxide and elemental iodine in different combinations were added.

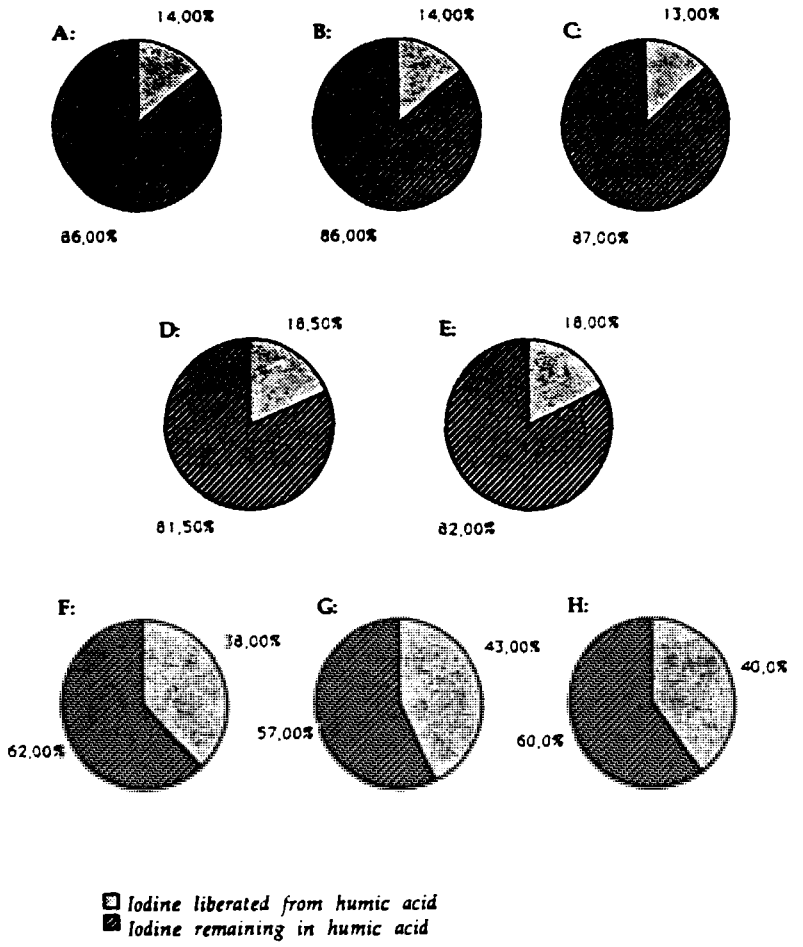


Fig. 17. Graphic representation of the distribution of bound and free iodine following deiodination experiments. Media A: acetate buffer, B: acetate buffer containing lactoperoxidase, C: acetate buffer containing lactoperoxidase and hydrogen peroxide, D: acetate buffer containing iodide, E: acetate buffer containing iodide and lactoperoxidase, F: acetate buffer containing iodide, lactoperoxidase and hydrogen peroxide, G: acetate buffer containing elemental iodine, and H: acetate buffer containing elemental iodine and lactoperoxidase.

It is noted that the results of the deiodination experiments are separated into three distinguishable groups. 1) A, B and C, where the iodinated humic acid were dissolved in acetate buffer, acetate buffer containing lactoperoxidase and acetate buffer containing lactoperoxidase and hydrogen peroxide, respectively. These three systems resulted in the release of approximately 14% iodine. 2) D and E, where the iodinated humic acids were dissolved in acetate buffer and iodide and iodide and lactoperoxidase, respectively, in which case an approximately iodine release of 18% was observed . 3) F, G and H where the iodinated humic acids were dissolved in acetate buffer containing iodide, lactoperoxidase and hydrogen peroxide, elemental iodine or elemental iodine and lactoperoxidase, respectively. For these three systems, the release of iodine was found to be approximately 40%.

These results suggest that possibly three different types of sites in the humic acid available for iodination are present: 1) sites where iodine is rather weakly bound to the humic acid structure, e.g. as π-complexes as formulated by Allinger et al. (1977), 2) sites susceptible for nucleophilic iodide-iodide substitution, a reaction type known to operate, e.g. in certain cases of production of iodinated radiopharmaceuticals (Mertens et al. 1987) and 3) sites susceptible for a conventional electrophilic iodine-iodine substitution, a reaction which has also been observed in the case of enzymatically controlled iodination of phenol. It should be noted that only a slight increase in the iodine release originating from the proposed nucleophilic aromatic substitution relative to the pronounced increase in the case where electrophilic aromatic substitution prevails, is in agreement with the general assumption concerning the possible operation of these two mechanisms (March 1968).

The above results suggest that the proposed equilibrium model fits the results of the equilibrium experiment but does not reflect the mechanism, and it does not appear to be the reaction rates of the forward and the reverse reaction that determine the degree of incorporation. Obviously, it could be the electrophilic iodine-iodine exchange which produces the picture of an

equilibrium.

7.8. Influence of pH

Huber et al. (1989) demonstrate that pH affected the iodination rate dramatically when peptides containing tyrosyl residues were iodinated applying lactoperoxidase as catalyst. Niedleman and Geigert (1986) stated that the optimal pH for the enzymatically controlled halogenation depend on the ratio of iodide to hydrogen peroxide (see page 47).

To elucidate the extent that the phenomena affected our results, enzymatically controlled iodination of humic acid was carried out in an acetate buffer of pH 4, pH 5 and pH 6. The incorporation was calculated after 10 min, 30 min and 4 hours, respectively. In Fig. 18 the incorporation of iodine into humic acid is plotted as a function of pH.

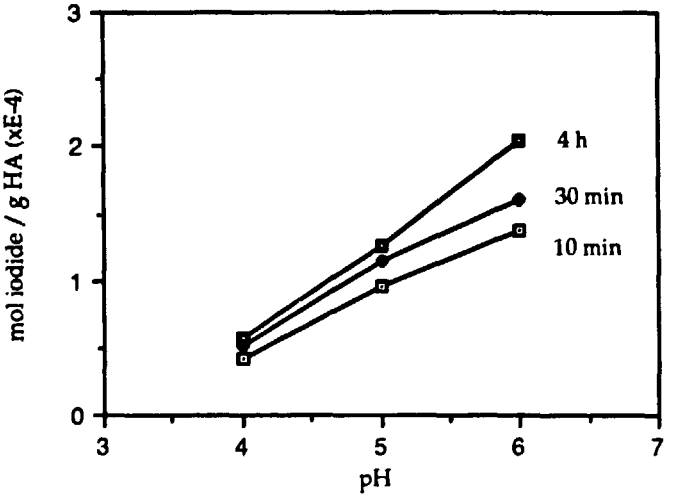


Fig. 18. Incorporation of iodine as a function of pH. (Initial concentrations: iodide, 5 x 10^{-5} mole/L; hydrogen peroxide 2 x 10^{-4} mole/L; humic acid, 0.1 g/L; lactoperoxidase, 10 µg/mL.

As can be seen from Fig. 18, quite different amounts of iodine were incorporated into humic acid under conditions of different pH. After 10 min

of reaction time, about 3 times as much iodine was incorporated at pH 6 as at pH 4. This tendency is unaffected by an increase in the reaction time.

In Fig. 19 different incorporations of iodine into humic acid obtained at pH 4, 5 and 6 are plotted as functions of reaction time.

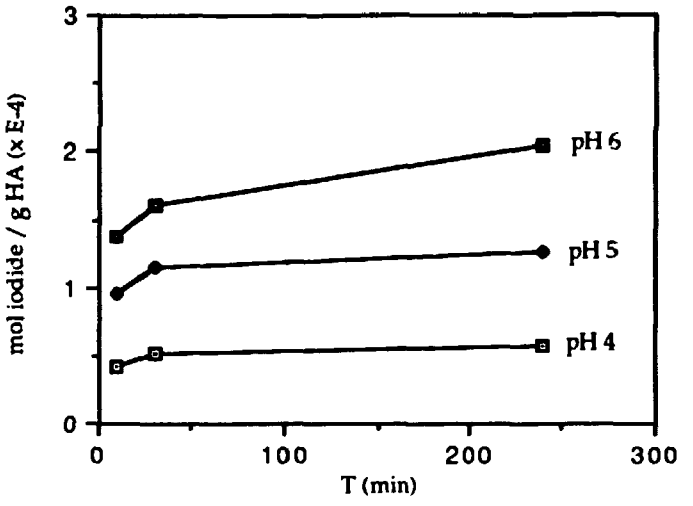


Fig. 19. Incorporation of iodine into humic acid as a function of reaction time (conditions as in Fig. 18.).

It is clearly demonstrated that the curves representing different pH level out, corresponding to different amounts of iodine incorporated into the humic acid. According to Huber et al. (1989), the total amount of incorporated iodine into peptides should be the same at different values of pH even when the reaction rates were different. The plot in Fig. 19 does not indicate that this is true when the accepter molecules are humic acids.

Huber et al. (1989) used 60 μM hydrogen peroxide and 400 μM iodide and found an optimal pH at about 6. Based on this result the optimal pH for equal concentrations of iodide and hydrogen peroxide can then be calculated as 5.18 according to the equation on page 47.

Morrison and Bayse (1970) found an optimal pH at 5 for a concentration of

hydrogen peroxide of 0.6 mM and that of iodide 3.5 mM. If one calculates the optimal pH for a concentration ratio of iodide to hydrogen peroxide (I^-/H_2O_2) of 1, one arrives at an optimal pH of 5.23, in pratice, this is identical to the one that was calculated using the results of Huber et al. (1989).

If we adopt an optimal pH at about 5.2 when the ratio of iodide to hydrogen peroxide is equal to 1, an initial optimal pH in our experiments can be calculated as 4.6 when I^-/H_2O_2 is 0.25. We found a great reduction of the incorporation of iodine when pH was changed from 5 to 4. During the consumption of iodide the optimal pH decreases further according to the equation which states that the optimal pH will change due to a change in the I^-/H_2O_2 ratio during the reaction when the initial I^-/H_2O_2 differed from 1.

Based upon these calculations and the levelling out of the incorporation, corresponding to different amounts of iodide incorporated at different pH, it seems unreasonable that the change in pH should be responsible for the lower level of incorporation due to a decreased reaction rate alone. An additional explanation seems to be necessary to explain the last results.

A deficiency of hydrogen peroxide and/or enzyme after about one hour could cause the observed effect if different reaction rates were operating at different values of pH. In our opinion another plausible explanation can be formulated: When pH decreases the protonisation of the humic acid increases and then the number of available sites will probably change. A combined effect may as well be responsible for the results, but it seems reasonable that fewer available sites at lower pH results in a lower incorporation.

7.9. Iodination of humic acid catalyzed by alternative peroxidases.

In accordance with the suggestion by Behrens (1982, 1985 and 1986) lactoperoxidase was used in al basic experiments. However, it seems more reasonable that enzymes formed from plants, fungi or microbes are located in

soil systems. Two types of enzymes representing plant peroxidases and peroxidases formed from fungi are commercially available: horseradish peroxidase (HRP) and chloroperoxidase (CP), the latter formed from the fungus *Caldiomyces fumago*.

By means of HPLC, the enzymatically controlled iodination of humic acid was demonstrated on applying horseradish peroxidase and chloroperoxidase. As can be seen from Fig. 20 the incorporation of iodine into humic acid was quite comparable to the results obtained by applying lactoperoxidase.

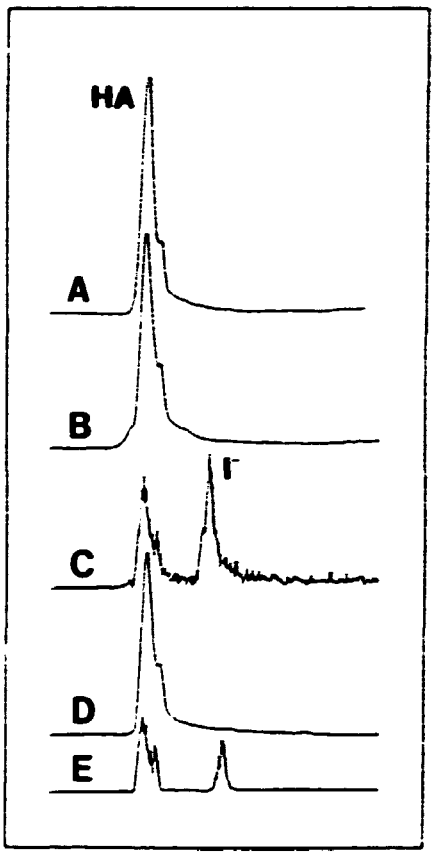


Fig 20. Chromatographic trace of A: humic acid prior to iodination (UV detection), B: humic acid after iodination with HRP (UV detection), C: humic acid after iodination with HRP (^{131}I detection), D: humic acid after iodination with CP (UV detection), and E: humic acid after iodination with CP (^{131} detection).

In order to demonstrate possible differences in the mechanisms involved in the iodination of humic acid catalyzed by LP, HRP and CP, experiments identical to the one leading to the equilibrium plot (Fig. 16) were carried out applying horseradish peroxidase and chloroperoxidase (fig. 21. and fig. 22.).

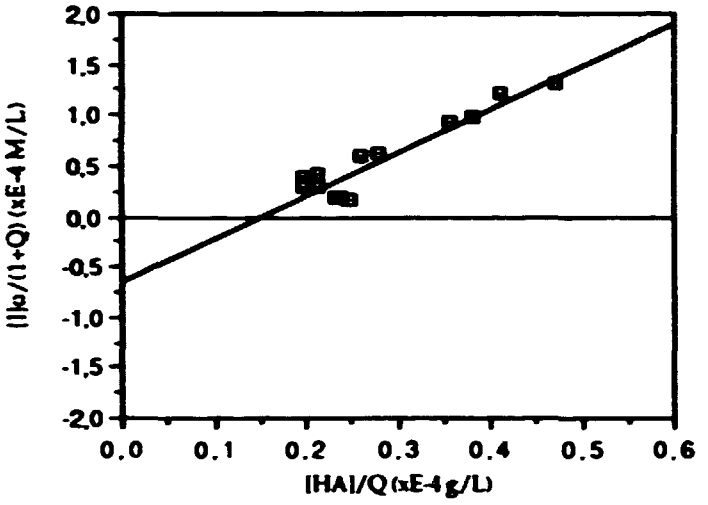


Fig. 21. Plot of initial iodide conc./(1+Q) vs. [HA]/Q for HRP-catalyzed iodination of humic acid.

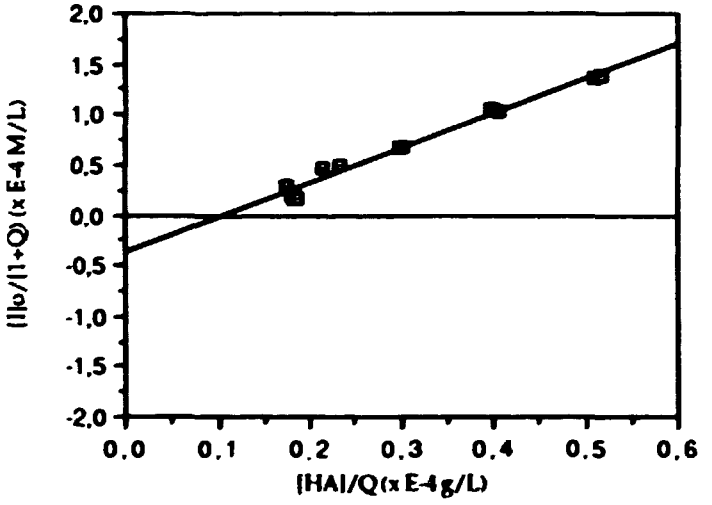


Fig. 22. Plot of initial iodide conc./(1+Q) vs. [HA]/Q for CP-catalyzed iodination of humic acid.

From the results visualized in Figs. 16, 21 and 22 it can be concluded that iodination of humic acid catalyzed by horseradish peroxidase and lactoperoxidase seem to be quite alike, while the equilibrium constant for the chloroperoxidase-catalyzed reaction seem to be greater and the number of sites a little lower. By definition the equilibrium constant ought to be identical independent of the peroxidase applied. However chloroperoxidase is known to catalyze the formation of IO_3^- from iodide, and if that reaction goes on too the concentration of the iodinating species becomes lower than expected. It seems acceptable that different amounts of sites are available for iodination if either the reaction goes on in a complex with the enzyme or if different iodinating species are formed. However, if the enzymes form identical iodinating species I_2 or HOI, then different numbers of sites available for iodination do not appear to be reasonable. Values for q and K_{eq} are listed in Table. 4.

Table 1. Number of sites, q (mol/g HA), available for iodination and equilibrium constants, K_{eq} (L/M), as a function of enzyme catalyst.

Enzyme	q	K_{eq}
LP	(4.28±0.22)x10-4	(1.32±0.17)x10⁴
HRP	(4.25±0.50)x10-4	(1.56±0.47)x10⁴
CP	(3.48±0.17)x10-4	(2.63±0.40)x10⁴

7.10. Naturally occurring iodir ation ability in soil.

To demonstrate that iodination of humic acid is catalyzed by naturally occurring substances which we believe happen to be peroxidases, we turned from commercial available enzymes to soil extracts.

Swedish soil from the same area near Varnamo as the soil used by Asplund et al. (*in press*) was extracted by the same procedure as used by these authors. This procedure was developed by Peterson and Kurylyak (1982) to extract peroxidases from soil, and Asplund et al. (*in press*) demonstrated that soil extracts produced this way were able to form di-chlorodimedone from mono-chlorodimedone in the presence of hydrogen peroxide and chloride.

We decided to use this spruce forest soil because it was already demonstrated that it exhibits halogenating capacity. Approx. 3.5 kg of soil were extracted and the extract concentrated from 4 L to 50 ml (see experimental section). Unfortunately, the soil extract still contained some humic substances and some of the DEAE which was used in the extraction procedure.

Experiments applying the soil extract as "enzyme" supply were carried out with an initial iodide concentration of 2.5×10^{-5} mole/L spiked with ^{131}I. The hydrogen peroxide was added stepwise over a period of 1 h 40 min as suggested by Asplund et al. (*in press*). The crude extract exhibited a remarkable ability to incorporate iodine into the high molecular weight fraction of the soil extract. About 40% of the iodide was transferred to the high molecular weight fraction. After 24 hours without further addition of hydrogen peroxide the percentage increased to about 50% (Fig. 23).

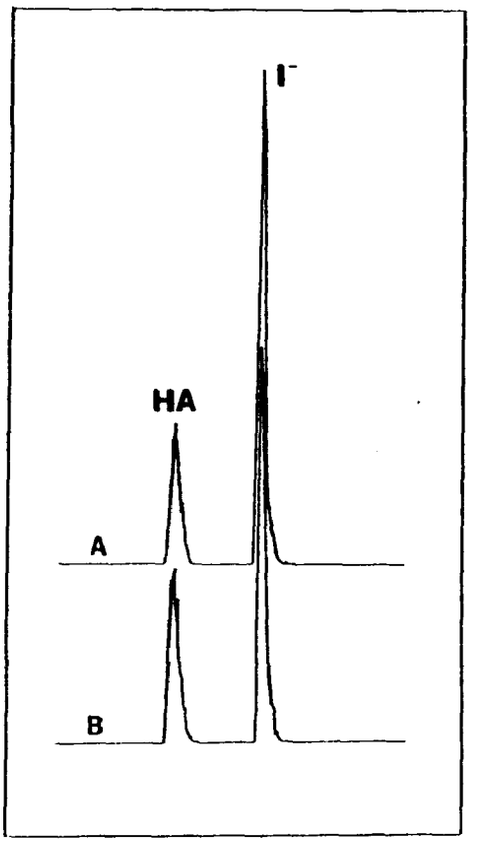


Fig. 23. Chromatographic trace of A: soil extract after 1 h 40 min of iodination, B: the same reaction mixture after 24 hours. Humic acid added 0.1 g/L. Hydrogen peroxide: 10 times 7.5×10^{-8} mol corresponding to a total concentration of 2.5×10^{-4} mol/L.

To remove undissolved substances the soil extract was centrifugated and the supernatant was filtered through a 0.45 μm filter, which turned the extract to a transparent yellow liquid. By means of experiments carried out with this extract it was demonstrated that the iodination capacity decreased dramatically by filtration. However, the iodination capacity still appeared and it was demonstrated that it was heat labile. After heating the extract to 90 °C for 15 min that activity was completely lost. When no hydrogen peroxide was added no incorporation did not take place and humic acid dissolved in acetate buffer containing iodide was not subject to iodination when hydrogen peroxide was added in a similar way in the absence of soil extract.

In the light of the work carried out by Behrens (1982,1985 and 1986), El Kekli and Johnson (1985) and others, our results seem to support the suggestion that extracellular haloperoxidases in soil are responsible for the iodination of humic substances in soil systems. We have demonstrated that three different haloperoxidases are able to catalyze the iodination of humic acid and that soil contains an "iodinating ability" which is heat labile, and which depends on the presence of hydrogen peroxide just as in the case of haloperoxidases.

8. THE IODINATION REACTION, A DISCUSSION

8.1. Verification

The results of the laboratory experiments seem at once to support the suggestion by Behrens (1985 and 1986) that extracellular enzymes of the peroxidase group catalyzes the iodination of humic substances under natural conditions. Raja and Babcock (1961) rejected their own suggestion of the involvement of microorganisms because the formation of organic iodine was not suppressed by 80% ethanol. However the iodination of phenol catalyzed by lactoperoxidase is uneffected when the enzyme has been dissolved in 80% ethanol for 10 min before it is added to the reaction mixture. The result obviously demonstrates that the conclusion by Raja and Babcock (1961) that microorganisms cannot be involved in the organic fixation of iodine does not preclude extracellular enzymes from playing a role.

The existence of a wide variety of extracellular enzymes in soil is well established (Burns, 1978) and methods for extracting peroxidases from soil have been developed (Bartha and Bordeleau, 1969), (Peterson and Kurylyak, 1982). Bollag et al. (1987) purified an enzyme from soil. Based on comparative studies of the purified enzyme and horseradish peroxidase, it was demonstrated that the enzyme exhibited the same substrate specificity as horseradish peroxidase and it was concluded that the enzyme was indeed a peroxidase.

In the present paper it has been demonstrated that horseradish peroxidase are able to catalyze the iodination of humic acid, and it seems reasonable to state that relevant extracellular enzymes occur in soil. However, it appears very difficult to demonstrate that the enzymes play the predominant role in the formation of iodinated humic acid under natural conditions.

In addition it has been demcnstrated that an "iodinating ability" can be extracted from soil and that this ability is heat labile and depends on the

presence of hydrogen peroxide just as peroxidases. It seems reasonable to postulate that the "iodinating ability" is identical to halogenating peroxidase, but in fact this can be only a suggestion.

In the following, the behaviour of iodine in the terrestrial environment will be discussed based on the assumption that the results of our laboratory experiments reflect processes going on in the soil and groundwater system.

8.2 The mechanism

According to Niedleman and Geigert (1986) there is still a great deal of confusion about the mechanism involved in the enzymatically controlled halogenation of organics. To simplify the problem of understanding this,only iodination will be discussed in this context. In most work dealing with enzymatically controlled iodination, tyrosine or peptides containing tyrosyl residues have been used as acceptor molecules for iodine. Only a limited research effort has dealt with other organic structures.

Niedleman and Geigert (1986) formulated two different mechanisms either of which may be involved in enzymatically controlled iodination:

1)

$$E + H_2O_2 \longrightarrow EO + H_2O$$

$$EO + I^- \longrightarrow HOI + E$$

$$AH + HOI \longrightarrow AI + H_2O$$

Where E = enzyme, EO = oxidized enzyme and A = organic molecule (mostly aromatic).

and a mechanism in which the reaction takes place on the enzyme

2)

$$E + H_2O_2 \longrightarrow EO + H_2O$$

$$EO + I^- \longrightarrow EOI$$

$$EOI + AH + H^+ \longrightarrow AI + H_2O + E$$

Bayse and Morrison (1971) demonstrated that elemental iodine (I_2) was formed as an intermediate in the reaction, an observation which is in agreement with our results. Then the reaction mechanism can be formulated as:

3)

$$E + H_2O_2 \longrightarrow EO + H_2O$$

$$\left.\begin{array}{l} EO + I^- \longrightarrow EOI \\ EOI + I^- \longrightarrow I_2 \end{array}\right\} \quad \text{or} \quad EO + 2I^- \longrightarrow I_2 + E$$

$$I_2 + H_2O \rightleftharpoons HOI + I^- + H^+$$

$$HOI + AH \longrightarrow AI + H_2O$$

In the second mechanism the iodinating species is formulated as a complex of the oxidized enzyme and iodide. The existence of this intermediate has not yet been demonstrated, but Morrison and Bayse (1973) concluded that due to the different iodination rates of D- and L-tyrosine the reaction has to take place on the enzyme. However Taurog (1970) and Huwiler et al. (1985) found no differences in the rate of lactoperoxidas-catalyzed iodination of D- and L-tyrosine.

Huber et al. (1989) studied the lactoperoxidase-catalyzed iodination of different peptides containing tyrosyl residues and demonstrated no

differences in rates of iodination. They showed also that iodination of large proteins occurred through a dialysis membrane and concluded that the iodinating species was formed by the enzyme in in the presence of iodide and hydrogen peroxide and then diffused from the enzyme. However, it is still possible that the reaction can follow two pathways: one takes place on the enzyme, the other proceeds via the formation of elemental iodine.

Niedleman and Geigert (1986) concluded that no results have been obtained which cannot be explained by hypohaleus acid chemistry. Our own experiments dealing with iodination of phenol did not demonstrate pronounced differences in product distribution either, suggesting different mechanisms for applying elemental iodine or peroxidases, iodide and hydrogen peroxide. For the moment the third mechanism seems to be the most probable.

8.3 What kind of sites are available for iodination in humic acid?

Some macromolecules have been subject to iodination catalyzed by lactoperoxidase. Marchalonis (1969) studied the enzymatically controlled iodination of immunoglubolins and other proteins. It was demonstrated that the ^{125}I was bound to tyrosyl residues. The iodine can be expected to attack ortho to the OH-group as in the iodination of tyrosine (Morrison and Schonbaum 1976). Millard (1988) demonstrated that plasmamembrane lipids were subject to enzyme-catalyzed iodination and Hemmaplardh and Morgan (1976) iodinated rabbit transferrin, but did not investigate what kind of substructures were iodinated.

According to the known examples of *in vitro* haloperoxidase-catalyzed iodination of organic molecules collected by Niedleman and Geigert (1986), only addition to double bonds in aliphatic compounds and electrophilic aromatic substitution ortho to OH-groups seem to occur. Also, Niedleman and Geigert (1982) demonstrated addition to alkynes.

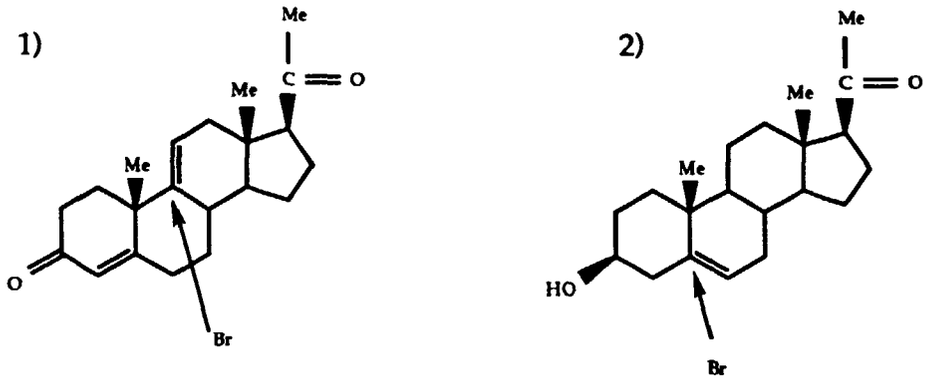
If the iodinating species is HOI, formed from elemental iodine and water, our own results suggest that the position between OH-groups meta to each other are available and that the position between an OH-group and the methyl-group as in orcinol is available too.

Benenson and Marcel (1978) demonstrated enzymatically controlled iodination of benzene dispersed in phosphate buffer.

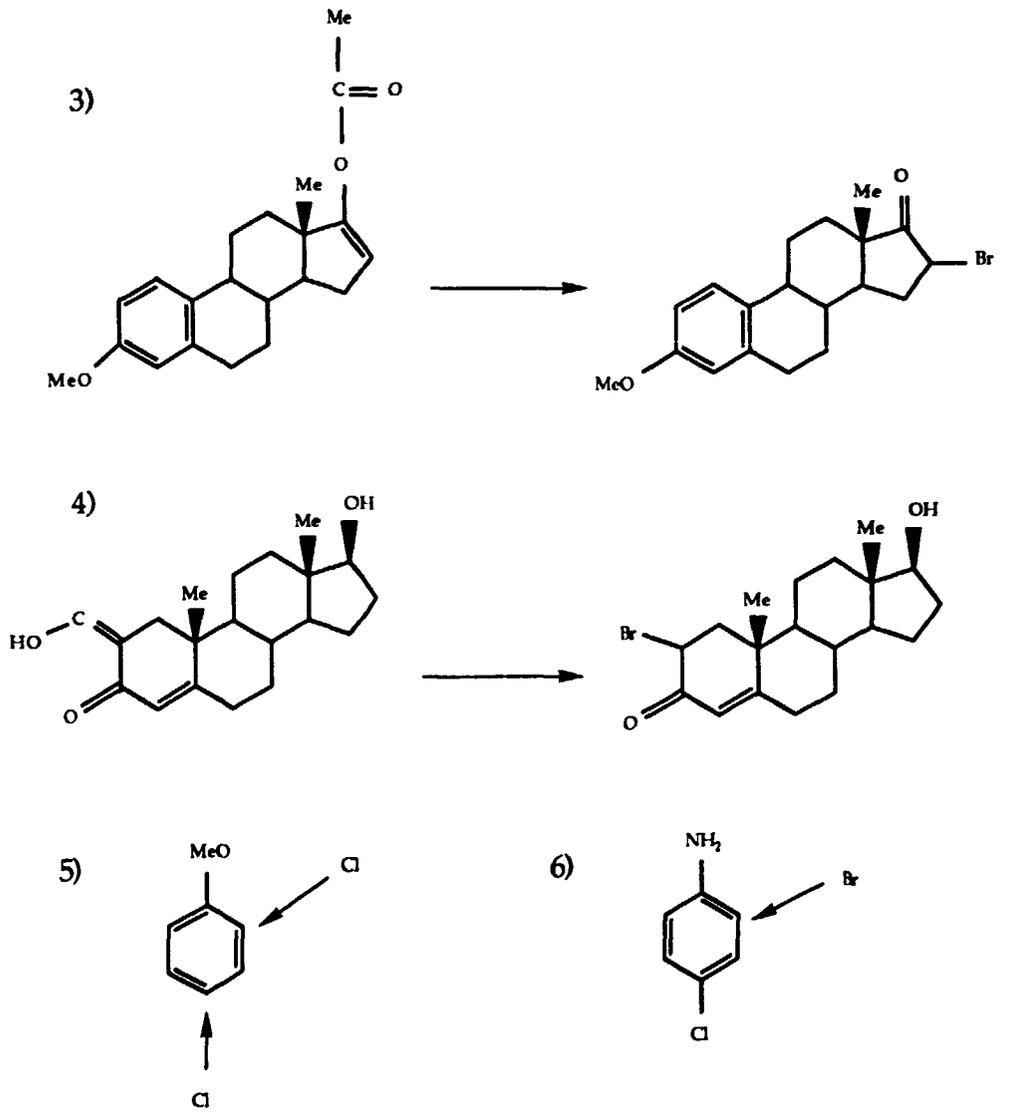
It is reasonable to believe that organic structures corresponding to those found to inhibit the function of the enzyme, *i.e.* resorcinol, will be subject to enzymatically controlled iodination when they are a structural part of a humic macro molecule. Humic acid was found to reduce the enzyme activity only partly, and obviously no substructures reduced the enzyme function completely in the concentrations that appeared. If elemental iodine is formed and hence HOI, iodination of all kinds of available sites will probably take place.

According to the "Compendium of *in vitro* haloperoxidase reactions" in Niedleman and Geigert (1986), only few investigations using iodide as halogen have been carried out with positive result. Addition to aliphatic alkenes forming halohydrines and halolactones and aromatic substitution to the phenol substituent in tyrosine have been demonstrated. Much more material is available about enzymatically controlled chlorination and bromination.

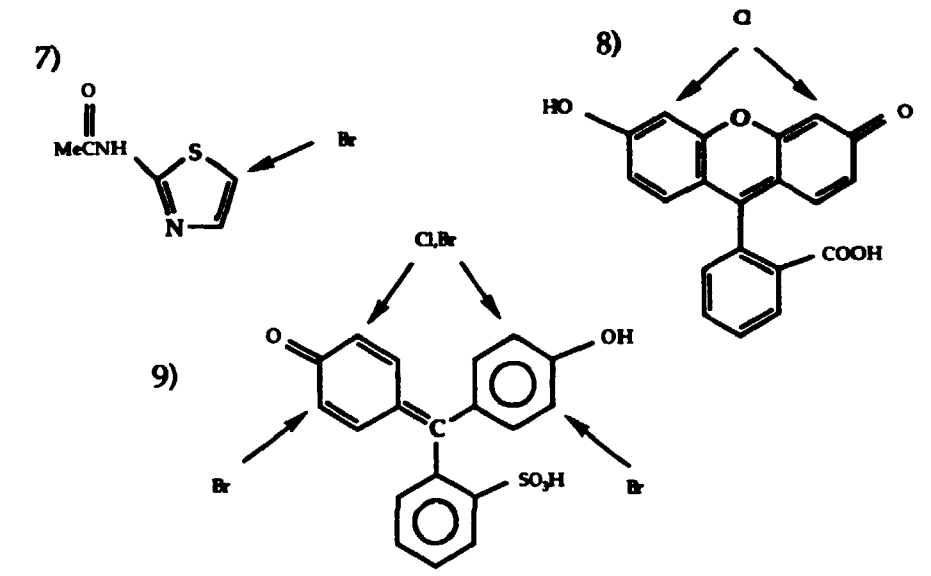
If one assumes that the sites in organic molecules available for chlorination and bromination are available for iodination as well, some additional types of sites may be involved too. Some molecules which can be chlorinated or brominated (Niedleman and Geigert, 1986) are listed below. Relevant parts of the molecules may exist as substructures in humic acid.



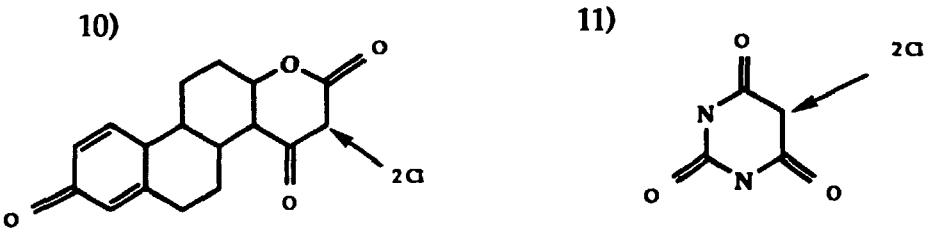
Forming alpha, beta halohydrins 1) and 2).



Electrophilic aromatic substitution 5) and 6).



Substitution reactions 7), 8) and 9).



Substitution to the alpha position in ß-diketones 10) and 11).

Harvey (1986) treated the organic matter from marine sediments with eleven chemical reagents and suggested that the content of iodine existed mostly in the electropositive state as N-iodoamides. The formation of iodoamides via HOI can be expected to proceed, corresponding to the formation of chloroamides as formulated by March (1968).

As one gathers from the above discussion, very little is known about the sub-structures in humic acid which can be iodinated by the function of haloperoxidases. However, the wide variety of substances that are able to be halogenated suggests that different kinds of sites are available. If the iodinating species is HOI and not an enzyme complex, structures located in

the "center" of the humic macro molecule may be iodinated as well as structures of the "surface"; this is suggested by the uniform distribution of iodine in humic acid shown by the HPLC trace in Fig. 3.

8.4 Equilibrium and reversibility

The statement by Niedleman and Geigert (1986) that haloperoxidase-catalyzed reactions are characterized by irreversibility seem at first to completely contradict our description of peroxidase catalyzed iodination of humic acid.

The experiments planned to elucidate the possible reversibility (page 52) demonstrated that some of the iodine is weakly bound to the humic acid and is easily released. We have formulated this in a preliminary way as being due possibly to the split of π-complexes. Fawcett and Kirkwood (1953) discussed the iodination of aromatic compounds in the light of rearrangement of the π-complex and if these π-complexes do exist with humic acids as the organic part, it is reasonable to expect that molecular iodine is released easily or transformed to iodide and hypoiodite by water.

However iodine can possibly be reversibly bound to sub-structures in humic acid that we have no knowledge about. In that case, the reverse reaction is independent of the enzyme because no enhanced release was observed in the presence of enzyme and hydrogen peroxide.

The pronounced electrophilic iodine-to-iodine exchange which was observed for iodinated humic acid can, however, be the key to an explanation as to why the reaction seems to be reversible. If the iodination rate is something lower than the rate of the electrophilic iodine-iodine exchange reaction, then the rate of further iodination will decrease dramatically when the concentration of iodinated sites increases at the expense of availability of free sites.

In Fig. 15 (page 48) it was demonstrated that different initial iodide

concentrations leads to different terminal amounts of iodine incorporated into the humic acid. If the reaction is not an equilibrium one, equal amounts of iodine should be incorporated into equal amounts of humic acid independently of the initial iodide concentrations as long as sufficient iodide is available. The explanation of the results must then be that the reaction is terminated before the maximum amount of iodine is incorporated.

The equilibrium plots suggest that the enzymatically controlled iodination of humic acid can be described as an equilibrium reaction. This is far from proved and different possible explanations of how it can appear to be an equilibrium reaction without actually being one are given above. Further investigations are necessary before the question of whether we are dealing with equilibrium or non-equilibrium can be answered.

9. INFLUENCE ON THE MIGRATION OF IODINE IN THE TERRESTRIAL ENVIRONMENT

According to Hamid and Warkentin (1967) ^{131}I has been regularly used for tracing water movement. It is generally accepted that stable iodide must be used as carrier to avoid adsorption to soil components and as demonstrated, lower concentrations of iodide will to a great extent be fixed in naturally occurring organic material.

Carlsen (1989) discussed the role of organics in the migration of radionuclides in the geosphere. It appears that complexation of metal ions by anthropogenic chelating compounds mobilize these metal ions and by that reduce the retention.

The addition of an anthropogenic complexing agent ethylenediaminetetraacetic acid (EDTA) enhanced the mobilization of iodine; however, only 4 - 20% of the fixed iodine was remobilized (Whitehead 1973a). It was argued that the explanation was to be found in the association of iodide with polyvalent metal ions. Thus, complexation of the metal ions released iodide to the liquid phase. Bors et al. (1988) demonstrated that nitrilotriacetic acid (NTA) and EDTA were able to remobilize iodine in soil. However, still only a few percent of the fixed iodine was mobilized.

The iodine seem to be fixed strongly in soil, but the fixed radioiodine may be remobilized by iodine-to-iodine exchange or by resolution of the precipitated iodinated humic acid. By iodine-to-iodine exchange, one may expect that with time the ratio of radioiodine to iodine will be the same in the humic acid as in the interstitial water. However, the equilibrium between dissolved and precipitated humic acid may also play the crucial role for the concentration of iodinated humic acid in solution.

Dehalogenation and breakdown of the humic acid may also be significant. Niedleman and Geigert (1986) devoted a chapter to the topic of biological

dehalogenation. Most work has been done inside the area of biological dechlorination of pesticides. A long list of microbes exhibiting dechlorination capacity has been produced. But in only in few cases has deiodination been given attention. Rosenberg and Goswami (1984) noted that 3',5'-diiodotyrosine was transformed to tyrosine by iodotyrosine deiodinase in the presence of NADPH. Scott and Sinsheimer (1984) demonstrated that rat liver preparations were able to deiodinate para-substituted aromatics including 4-iodophenol.

The C-I bond shows the lowest bond energy of the carbon-halogen bonds (46 kcal/mole). If biological dechlorination takes place in the terrestrial environment one may expect deiodination to take place as well. However, the existence of biological deiodination in the terrestrial environment has to be demonstrated and the role further investigated.

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APPENDIX 1

MATERIAL AND METHODS FOR EXPERIMENTS UNAVAILABLE ELSEWHERE

Resistance of lactoperoxidase to 80% EtOH

Chemicals: NaI (Merck p.a.), ^{131}I as NaI, lactoperoxidase (EC.1.11.1.7.) (Sigma L2005), H_2O_2 (J.T. Baker Chemicals),phenol, acetate buffer pH 5 and ethanol 96%.

Lactoperoxidase was dissolved in 80% ethanol for 10 min and then added (2µg/mL) to a solution of iodide, phenol and H_2O_2 all concentrations being 10^{-4} M in acetate buffer pH 5. The reaction mixture was analyzed by means of HPLC.

Iodination of phenol in excess of phenol by I_2 or HOI

Chemicals: NaI (Merck p.a.), I_2 (Merck, p.a.), phenol, Naoh (merck p.a.), acetatebuffer pH 5.

1 mL I_2 - solution (10^{-3} M) and 1 mL NaOH (10^{-3} M) were mixed and subsequently 1 mL acetate buffer pH 5 and 1 mL phenol (10^{-2} M) were added. After 20 min the product was analyzed directly by means of HPLC. The experiment was repeated but instead of NaOH distilled H_2O was added.

Iodination of humic acid by I_2

Chemicals: NaI (Merck p.a.), KIO_3 (Merck p.a.), ^{131}I as NaI, HCl (FERAC zur analyse), NaOH (Merck p.a.), Humic acid (Aldrich) and acatate buffer pH 5.

200 µL NaI (spiked with ^{131}I) (2.5×10^{-3} M), 50 µL KIO_3 (4×10^{-4}) and 50 µL HCL

(0.1 M) was mixed until brown colour. 4300 µL NaOH (1.15×10^{-3} M) was added and subsequently 300 µL acetate buffer pH 5. 100 µL humic acid (5 mg/mL) was then added and after 20 min the reaction mixture was analyzed directly by means of HPLC.

Enzymatically controlled iodination of humic acid (pH variation)

Chemicals: Humic acid (Aldrich), H_2O_2 (Merck p.a.), NaI (Merck p.a.),^{131}I as NaI, Lactoperoxidase (EC.1.11.1.7.) (Sigma L-2005), acetate buffer pH 4, 5 and 6, NaOH (Merck p.a.), $NaHSO_3$ (J.T. Baker Chemicals) and HCL (FERAC zur analyse).

In a total volume of 5 ml (acetate buffer pH 4, 5 and 6), iodide (spiked with ^{131}I) and humic acid were mixed. The reaction was initiated by addition of lactoperoxidase and hydrogen peroxide. The reactions were allowed to proceed for 10 min, 30 min and 4 hours at the three different pH-values and were terminated by addition of 1 ml of $NaHSO_3$ (0.1 M). The reaction mixtures were transferred to new vessels in order to eliminate iodine adsorbed to the internal surfaces of the orriginal reaction vessels. 1 ml of HCL (12 M) was added. The reaction mixtures were alowed to rest for 5 min and were subsequently centrifugated for 25 min (3000 rpm). 4 ml of the supernatant plus 1 ml of water were subjected to gamma counting. 2 ml of NaOH (2M) were added to the vessels containing the precipitated humic acid and the rest of the supernatant to dissolve the precipitated material. The solutions were subsequently subject to gamma counting and the distribution of iodine in the humic acid and in solutions was calculated based on the mutual counting rates. The initial concentrations were: iodide, 5×10^{-5} mole/L; H_2O_2, 2×10^{-4} mole/L; humic acid , 0.1 g/L and lactoperoxidase 10µg/mL.

Naturally occurring iodination ability

Chemicals: Cellulol DEAE type SF (Biochemical Corporation) and KCL

(Merck p.a.), HCL, NaOH, NaI, $NaHSO_3$, humic acid and H_2O_2 as above.

Extraction of "enzymes" from soil

Soil (3.5 kg) was collected in October in a spruce forrest 4 km north of Varnamo in Sweden. The soil was sifted through an 8-mm sieve and extracted by 4 L of phosphate buffer pH 3.6 containing KCl (2 mole/L). After 15 min the mixture was filtered through a Whatman filter no. 3. To the mixture was added 100 g activated DEAE (se belove); it was stirred for 30 min and subsequently filtered through a Whatman filter no. 3. The filtrate was dialyzed (MW cut of 12,000) against distilled water and concentrated in an ultraconcentration cell (Amicon, model 8400, equipped with a YM-10 filter, MW cut of 10,000) to 1 L. During the process the temprature was kept at about 3 °C. The concentrate was further concentrated by freeze-drying to dryness and dissolved in 50 mL distilled water.

Activation of DEAE

DEAE (100 g) was dispersed in 1 L NaOH (0.3 M) and filtered through a Whatman filter no. 3. Then the DEAE was dispersed in 1 L of HCL (0.5 M) and filtered through a Whatman no. 3. The DEAE was finally dispersed in distilled water and filtered and washed until a neutral reaction in the wash water was reached.

Verification of the iodinating ability in soil

In a total volume of 3 mL, 2 mL soil extract was mixed with iodide (2.5×10^{-5} mole/L) (spiked with ^{131}I), acetate buffer pH 5 and humic acid (0.1 g/L). Hydrogen peroxide was added over a period of 1 h 40 min, 10 times 7.5×10^{-8} mole corresponding to a total addition of 2.5×10^{-4} mole/L. The reaction mixture was analyzed directly by means of HPLC. The column applied was a 250 x 4.6 mm Ultrahydrogel™; the eluent was 0.1 M sodium acetate adjusted to pH 9.6, and the flow was 0.6 mL/min. The reaction mixture was analyzed

again after a 24-hour rest. Corresponding experiments were carried out after the soil extract had been filtered through a 0.45 μm filter. In addition, two experiments were carried out; one without adding of hydrogen peroxide and the other after heating the soil extract to 90 °C for 15 min.

APPENDIX 2

DISCUSSION OF THE EXPERIMENTAL METHOD

Iodination of phenol and the antithyroid compounds

A direct analysis of the reaction mixture was made by means of HPLC. The relative amounts of the different iodophenols formed were calculated from the area of the single peaks in the chromatogram and the relative number of iodine atoms per molecule. The method seems to satisfy the demand of a rapid analysis of the product distribution in a great number of product solutions. The use of ^{131}I-spiked iodide ensured that only iodine-containing species were taken into account in the calculation. Additional analyses by means of MS only served to confirm that the expected masses occurred.

Analyses of the products formed by iodination of the "antithyroid compounds" were carried out by means of MS. As above, it was possible to detect only the masses corresponding to the iodinated forms of the original molecules, and different isomers were not identified. However, in some cases when only one type of site was present or if poly-iodinated compounds were formed it was possible to suggest a reasonable structure of some of the compounds in the product mixtures.

It would have been reasonable to analyse the products formed from iodination of the "antithyroid compounds" as in the case of phenol. However, the authentic iodinated compounds were not commercially available, and quite different chromatographic conditions were necessary to analyse the products. For those reasons it was decided that mass identification to an acceptable degree was satisfactory.

Iodination of humic acid

Analysis of iodinated humic acid by means of HPLC satisfied our demand of

verification. It was clearly demonstrated that the iodine was strongly adsorbed to the humic acid fraction. However, some of the compounds containing iodine were washed out of the column only slowly leading to a general decreasing signal to noise ratio in the radioactivity signal. The chromatographic peak of humic acid showed a long tail and because the iodide peak was eluted quite close to the humic acid the two fractions were not separated satisfactorily enough for quantitative calculations.

To obtain quantitative results precipitation of the iodinated humic acid was necessary. Precipitation of humic acid under alkaline conditions by calcium ions was quite effective. Unfortunately some inorganic compounds containing iodine were precipitated too. Acid precipitation required a longer time of centrifugation (25 min instead of 10 min) to get the humic acid satisfactory precipitated. When the method was changed to involve acid precipitation of the humic acid, the method to stop the reaction was changed to addition of $NaHSO_3$ instead of NaOH. Addition of $NaHSO_3$ makes it probable that no elemental iodine exists, while NaOH could precipitate the formation of other iodinating species. The third problem was that the single use tubes which were used in the gamma counting system were made of polyethylene. Elemental iodine migrates into this material and during the reaction some of the iodine was fixed in the wall of the tubes. The per cent of the iodine which was fixed in the polyethylene increased with the concentration of iodide. This problem was overcome by stopping the reaction in the reaction tube by addition of $NaHSO_3$ and then transferring the mixture to a new tube. The iodine fixed in the reaction tube was then subject to gamma counting and the amount of fixed iodide calculated.

Only in the equilibrium experiments may the fixation of iodine in the tube wall distort the values obtained by calculation. However, it is only in the case of high iodide concentrations that substantial amounts of iodide are fixed in the wall of the tubes.

The calculation can be made by taking two different assumptions into

account: A) The fixed iodine can be accepted as free iodide or B) it can be excluded from the calculation, corresponding to a lower initial iodide concentration. In Fig. 24 the equilibrium plots of lactoperoxidase are shown for both types of calculations.

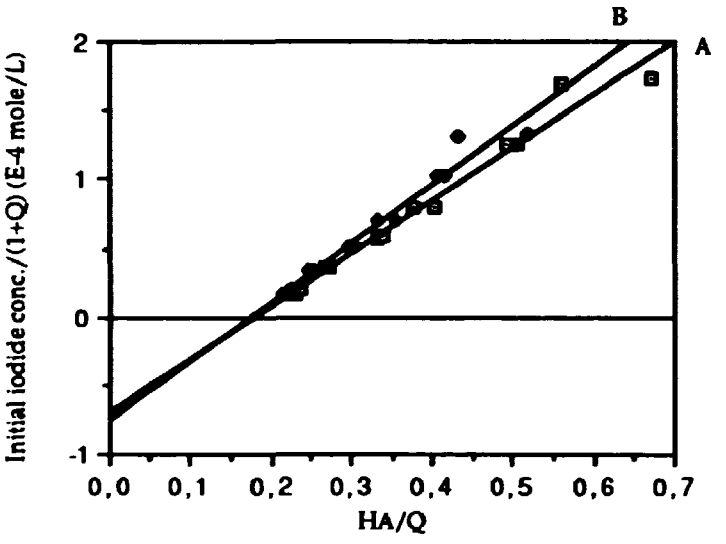

Fig. 24. In curve A the iodination fixed in the tube is taken into account as free iodide. In curve B the amount of iodide fixed in the tube is excluded from the calculation corresponding to a lower initial iodide concentration.

As can be seen from the curves, the results change only slightly, the number of sites are in case A) 3.86 x 10^{-4} mole s/g HA and in case B) 4.30 x 10^{-4} mole s/g HA. The equilibrium constant K_{eq} changes from 1.33 x 10^4 L/mole in case A) to 1.44 x 10^4 L/mole in case B).

If the results do reflect an equilibrium situation the true equilibrium constant and the number of sites may be in the range between the results of these two calculational methods.

From the results involving enzyme-catalyzed iodination of humic acid the following two questions arise: Does the enzyme preserve the activity? Will a deficiency of hydrogen peroxide take place as the reaction proceeds? A detailed knowledge of the answers to these two questions is necessary if the

topic shall be further elucidated. As demonstrated on page aa the pH seems to affect the number of sites which are available and the dependence of pH may also be subject to further investigations.

In the experiments involving naturally occurring "iodination ability", there is of course no guaranty that it is the enzymes which are responsible for the iodination. Isolation of the appropriate enzymes as was done by Bollag et al. (1987) is necessary if the iodination of humic acid by soil enzymes shall be unambiguously demonstrated.

However, if the enzyme-catalyzed iodination of humic acid shall be demonstrated as the major mechanism for retention of iodine in soil systems, selective peroxidase-inhibiting compounds must be demonstrated to reduce or completely inhibit the retention ability of iodide in soil.

APPENDIX 3

DERIVIATION OF THE EQULIBRIUM EQUATION (page 50).

$$S + "I" \rightarrow SI$$

1) $[SI]/[S]["I"] = K_{eq}$
2) $[I]_o = [SI] + ["I"]$
3) $[S]_o = [SI] + [S] = q[HA]$
4) $Q = [SI]/["I"]$

according to 1) $[S] = Q/K_{eq}$

according to 4) $[SI] = Q["I"]$

$$q[HA] = Q["I"] + Q/K_{eq} \Longrightarrow$$

$$Q["I"] = q[HA] - Q/K_{eq} \Longrightarrow$$

$$["I"] = q[HA]/Q - 1/K_{eq}$$

$$[SI] + ["I"] = [I]_o \Longrightarrow$$

$$Q["I"] + ["I"] = [I]_o \Longrightarrow$$

$$(1+Q)["I"] = [I]_o \Longrightarrow$$

$$["I"] = [I]_o/(1+Q) \Longrightarrow$$

$$[I]_o/(1+Q) = q[HA]/Q - 1/K_{eq}$$

where

["I"] = concentration of the iodinating species put equal to the conc. of iodide.

$[I]_o$ = the initial iodide conc.

[S] = the conc. of uniodinated sites.

[SI] = the conc. of iodinated sites.

[HA] conc. of humic acid in g/L.

q = the number of sites in moles/g HA.

K_{eq} = the equlibrium constant.

S_o = the number of sites in a given amount of humic acid.

97/98

APPENDIX 4

IODINATION OF PHENOL

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IODINATION OF PHENOL

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ABSTRACT

Phenol is iodinated in aqueous solution at pH 5 (acetate buffer) by elemental iodine or, if the iodine is present as iodide, enzymatically controlled by peroxidases. Generally mono-, di- and triiodophenols are obtained, the overall product composition being virtually identical for the two iodination modes. However, there is a tendency to a higher *para* to *ortho* ratio for the enzymatically controlled reaction. The mutual ratios of the single iodophenols depends on the initial concentration ratio between phenol and the iodinating species. The first step in the iodination leads preferentially to substitution in the *ortho* position rather than in the *para* position in contrast to *e.g.* the corresponding bromination. The relative rates of the competive reactions in the combined iodination scheme has been derived.

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1. INTRODUCTION

For many years major attention to the environmental cycle of iodine has been paid due to its status as essential element to man. In recent years, after the introduction of nuclear power as an energy source, the environmental cycle of iodine has received renewed interest due to the possible release of the longlived radioactive ^{129}I isotope, *e.g.* during reprocessing.[1] Man concentrates iodine in the thyroid gland. Hence, high ^{129}I levels in the environment, and subsequently high levels in the human thyroid gland may cause servere damages, *i.e.* development of cancer.

It is generally believed that iodination of tyrosin in the thyroid gland is a reaction, which is catalyzed by an enzyme of the peroxidase group, "thyroid peroxidase", in the presence of hydrogen peroxide.[2] Thus, it seems resonable to assume that enzymes of the peroxidase group are able to catalyze the iodination of phenols by iodide in the presence of hydrogen peroxide.

As a preliminary study to investigations on the possible enzymatically mediated iodination of naturally occurring phenolic compounds, as humic substances, in the terrestrial environment, we report in the present paper on the iodination of phenol by elemental iodine and by iodide in the presence of hydrogen peroxide applied lactoperoxidase (LP) as the enzyme catalyst.

2. EXPERIMENTAL

2.1. Chemicals

Phenol (*p.a.*) and 2-iodophenol (*p.a.*) were obtained from Merck. 4-iodophenol (99%) and 2,4,6-triiodophenol were obtained from Aldrich-Chemie. NaI (Merck p.a.), ^{131}I as NaI, Lactoperoxidase (EC.1.11.1.7.) (Sigma L-2005), Acetate buffer pH 5, $NaHSO_3$ (J.T. Baker Chemicals) and HCl (FERAC zur analyse).

2.2. Preparation of iodophenols by direct iodination (by I_2) of phenol

A solution of 9,4 g (0,1 mole) phenol and 28 g (0,11 mole) I_2 in 1000 mL acetatebuffer (pH 5) were stirred for 50 h. Remaining iodine was reduced with 55 mL 0.4 M NaS_2O_3. The solution was extracted with 4 x 125 mL diethyl ether. The combined organic layers were washed with 4 x 100 mL 0,1 M $NaHCO_3$, dried (Na_2SO_4) and evaporated to an oily substance under reduced pressure. The diiodophenols were separated by means of preparative HPLC applying a 250x16 mm Lichrosorb RP18 (5 µm) column (Eluent: MeOH/H_2O 70/30 v/v (33 min) and subsequent pure MeOH (3 min), Flow rate: 5 mL/min, UV-detec.: 280 nm).

2.3. Variation of the initial phenol concentration

In a total volume of 10 mL acetatebuffer pH 5 , phenol, NaI, H_2O_2 and lactoperoxidase were allowed to react, the NaI being spiked with ^{131}I. Initial concentrations of NaI and H_2O_2 were 10^{-4} M and 10 µg lactoperoxidase (E.C. 1.11.1.7.) corresponding to aproximately 0.8 units were used. The phenol concentration was varied in the range from 3.3 x 10^{-5} to 2 x 10^{-4} M. After mixing phenol, NaI and H_2O_2 the reactions were initiated by addition of the enzyme and were allowed to proceed for 10 min. at ambient temperature. The solutions were analyzed by means of HPLC without further treatment applying a 250x4.6 mm Nucleosil C8 (10 µm) column (Eluent: MeOH/H_2O 50/50 v/v, Flow rate: 1 mL/min). Control experiments without applying lactoperoxidase were carried out analogously.

2.4. Enzymatic iodination of ^{14}C-phenol

In a total volume of 10 ml acetatebuffer pH 5 phenol at a conc. 3.3 x 10^{-5} (spiked with ^{14}C-phenol) was enzymatically iodinated as described above (*cf.* 2.3.).

2.5. Experiments investigating the rates of the respective iodination reactions

Phenol and iodophenols were iodinated as single species as well as in combination, the procedure being as outlined above, however, applying phenol and/or iodophenols in ten-fold excess compared to iodide. The following system were studied: a) phenol, b) phenol + 2-iodophenol, c) phenol + 4-iodophenol, d) 2-iodophenol, e) 4-iodophenol, f) phenol + 2,6-diiodophenol, g) phenol + 2,4-diiodophenol, h) 2,6-diiodophenol and i) 2,4-diiodophenol. The concentrations of the phenols were 1×10^{-4} M whereas iodide and hydrogen peroxide concentrations were kept at 1×10^{-5}M. Lactoperoxidase concentrations were 10 µg/10 ml. The reaction mixture were analyzed by means of HPLC (*vide supra*).

2.6.Comparison of iodination and bromination of phenol applying elemental iodine and bromine, respectively

In a total volume of 50 mL (acetate buffer, pH 5) phenol (10^{-2} M) is reacted with the appropriate elemental halogen (10^{-3} M). Excess of phenol is used in order to avoid consecutive halogenation of the primary formed monohalogenated phenols. After complete decolorization of the reaction mixture pH was ajusted to 1 (HCl). The reaction mixture was extracted with diethyl ether (2x100 mL). The combined organic layers were dried (sodium sulphate) and evaporated to dryness. The product was dissolved in 1 mL pyridine and hexamethyl disilazane (250 µL) and trimethyl chlorosilane (250 µL) were added.[3] The mixture was centrifugated and the supernatant subjected directly to gas chromatographic analysis, applying a 30 m x 0.52 mm capillary DB-1 coloum (150°C isotherm./FID).

2.7. Iodination of phenol by iodide and chloramine-T

To 1 mL H_2O containing 0.220 g chloramine-T was added 1 mL H_2O containing 0.118 g NaI (spiked with ^{131}I). After 2 min 1 mL H_2O containing

0.062 g phenol was added to the mixture. After 1 h 10 mL of 50% MeOH was added, and the reaction mixture was analyzed directly by means of HPLC.

The experiment was repeated without ^{131}I. The 3 mL of reaction mixture was added to 10 mL H_2O, and extracted with 25 mL ethylacetate. The organic layer was dried (Na_2SO_4) and evaporated to a volume about 2 mL. The product was analyzed by means of MS.

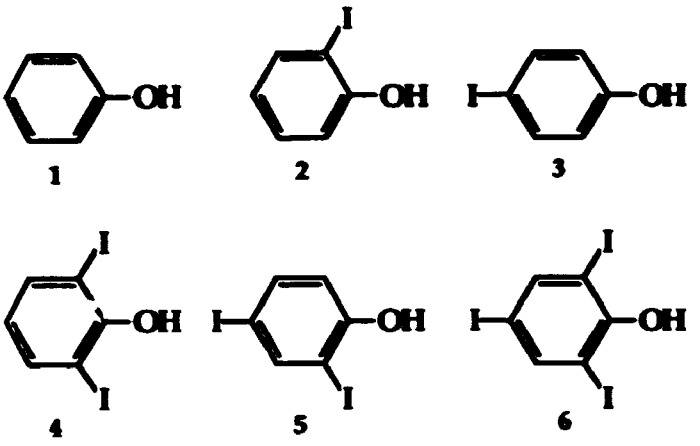
2.8. Reaction of mono-iodophenols with chloramine-T

Chloramine-T (7 mg) was added to 25 mL of 10^{-3} M aqueous solutions of 2-iodophenol or 4-iodophenol, respectively. The mixtures were stirred for 1 h, extracted with 25 mL ethylacetate. The organic layer was dried (Na_2SO_4) and evaporated to a volume about 2 mL. The products were analyzed by means of MS.

3. RESULTS AND DISCUSSION

3.1. Iodination by aqueous iodine and by iodide mediated by lactoperoxidase

The direct iodination of phenol (1) by aqueous I_2 turned, not unexpected out to give five different iodophenols: 2-iodophenol (2), 4-iodophenol (3), 2,6-diiodophenol (4), 2,4-diiodophenol (5) and 2,4,6-triiodophenol (6). The single compounds were indentified by comparison with authentic compounds. The product distribution was in accordance with the combined electronic effect of the OH-group (activating positions 2 and 4) and iodine (deactivating positions 2 and 4). When phenol is iodinated in dilute solutions (10^{-4} M) of aqueous iodine, nearly all the iodine was consumed within few minutes forming iodophenols.



Iodide does not react with phenol in the absence of an oxidizing agent.

Operating in relatively high concentrations of iodide and hydrogen peroxide, elemental iodine is formed in concentrations sufficiently high to interact with the phenol, possibly *via* a primary formation of hypoiodous acid (HOI) as the actual iodinating species (*vide infra*). However, for I^- and H_2O_2 concentrations below ca. 10^{-4} M apparently no iodophenols could be detected within 10 min. Hence, the enzyme catalysis may play a crucial role in the possible iodination of phenol in the low concentration ranges as *e.g.* expected in the environment. It is in this context worthwhile to note that the enzymatically catalyzed iodination *a priori* will lead to 100% consumption of the iodide in the reaction mixture, whereas, in the possible absence of an oxidizing agent, only a 50% consumption, as a maximum, can be expected using elemental iodine/hypoiodous acid as iodinating species, leaving the remaining 50% as iodide.

HPLC analysis of the products from the enzymatically mediated iodination of phenol showed no discrepancies compared to that originating from the direct iodination. Enzymatic iodination of phenol using ^{131}I and ^{14}C-phenol, respectively, showed ^{131}I and ^{14}C labeling at the same five positions in the HPLC trace, strongly indicating that the same 5 iodophenols were produced (fig. 1 & 2).

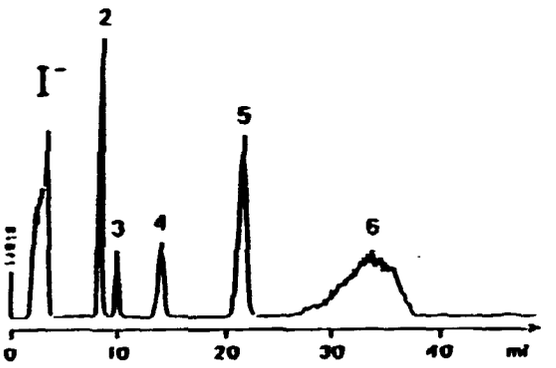


Fig. 1. HPLC trace of the product from enzymatic iodination of phenol using ^{131}I, detecting the radioactivity signal.

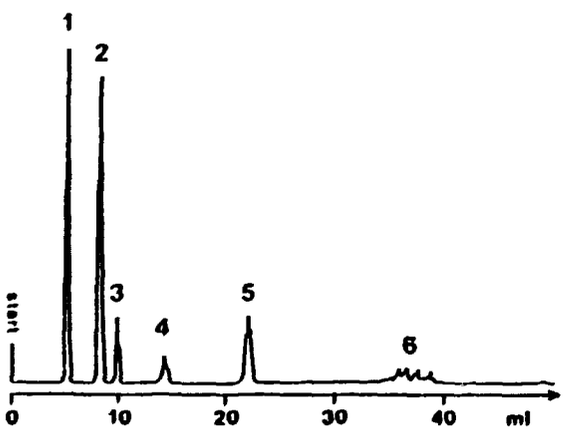


Fig. 2. HPLC trace of the product from enzymatic iodination of phenol using ^{14}C phenol, detecting the radioactivity signal.

For fixed initial I^- concentrations equal to 10^{-4} M and varying phenol concentrations (3.3×10^{-5}, 5.0×10^{-5}, 1.0×10^{-4} and 2.0×10^{-4} M), the relative yields of the single iodophenols are elucidated by HPLC analysis as visualized in fig. 3.

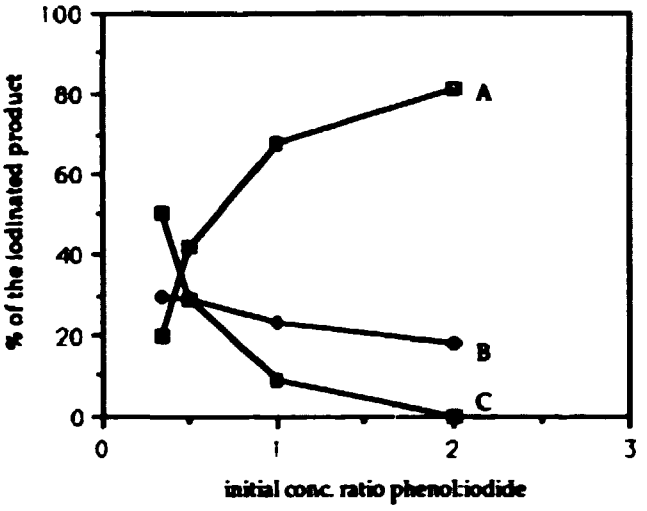
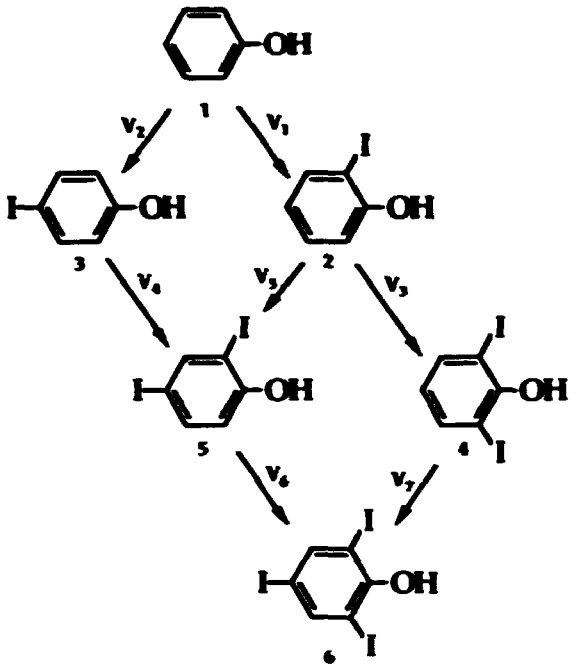


Fig. 3 The percentage of A: mono-, B: di-, and C: triiodophenols among the products originating from enzymatic iodination of phenol as a function of the initial phenol:iodide concentration ratio.

It is immediately seen (fig.3) that monoiodophenol, not surprisingly, dominates when the initial phenol/I^- concentration ratio is relatively high and that the degree of di- and tri-iodination increases with decreasing initial phenol/I^- ratio. Thus, since the enzymatic iodination does not give rise to other isomers than does the direct iodination, *i.e.* by elemental iodine, of phenol, the iodination reaction obviously can be formulated as series of consecutive steps from phenol to mono-, di-, and tri-iodophenol, controlled by concentrations of reactants as well as the mutual rate constants.



Scheme 1

In order to elucidate the relative rates of the "competitive" iodination reactions different combinations of phenol and iodophenols were iodinated enzymatically, the phenols being applied in large excess. Thus, only the first iodo-derivatives from the parent molecules are formed. Taking into account that iodination of phenol may lead to 2- and 4-iodophenol, the probability of producing the former being twice that of the latter, it could be concluded that 2-iodophenol is produced with a rate approximately three times faster than 4-iodophenol. The relative reaction rates, based on product distributions, are given in Table 1.

Table 1. Relative rate relations of iodination based on product destribution.

Reactants	rate relation
Phenol,	$V_1 > V_2$
2-iodophenol	$V_3 > V_5$
Phenol, 2-iodophenol	$V_3 \geq V_1 + V_2$
	$V_5 \sim V_1 + V_2$
Phenol, 4-iodophenol	$V_4 \sim V_2$
Phenol, 2,4-diiodophenol	$V_7 >> V_1$
Phenol, 2,6-diiodophenol	$V_6 << V_1$

Iodination of 2-iodophenol lead to 2,6- and 2,4-diiodophenol in the ratio approx. 1,7 : 1. Iodinating mono-iodophenols in combination with phenol and calculating the rates relative to the iodination rate of phenol, lead to the conclusion that the 6-position in 2-iodophenol was about equal as favourable as the original 2-position in phenol. On the other hand, the 2- and 6-position in 4-iodophenol surprisingly appeared significantly less favorable for iodination. However since we operate in relatively high concentrations of the phenols (10^{-4}), it cannot be excluded that the relative rates determined under these conditions are influenced by an interaction between iodophenols and the enzyme reflecting an inhibiting tendency. Enzymatic iodination of 2,6-diiodophenol and 2,4-diiodophenol caused formation of 2,4,6- triiodophenol. However, when phenol is available as substrate, 2,4-diiodophenol will only to a very minor degree, if at all, be iodinated. In contrast the rate of forming 2,4,6-triiodophenol from 2,6-diiodophenol

appeared significantly higher than that of forming monoiodophenol from phenol.

From the relative reaction rates it can be concluded that in general the activation of the carbon-atoms for further iodination increases with degree of iodination. Activation of the actual C-atoms appears to be: phenol < mono-iodophenol < diiodophenol, except in the case where the 4-position is occupied by iodine. An explanation may be a possible interaction between the enzyme and the iodine atom located in 4-position, since the 4-position in 2,6-diiodophenol is very favourable for further iodination. However, an enzymatically controlled iodine exchange of iodine in the 4-position may also cause the observed effect.

When 2,4-diiodophenol is enzymatically iodinated applying ^{131}I labelling, ^{131}I labelled 2,4-diiodophenol is formed. The exchange reaction does not proceed in the absence of enzyme. Further more, it was observed that 2,6-diiodophenol shows no exchange reaction. Upon iodination of 4-iodophenol applying ^{131}I minor amounts of ^{131}I labelled 4-iodophenol was noted. Hence, it appears obvious to conclude that only the iodine in the 4-position is subject to enzymatically controlled iodine exchange, possibly *via* a simple electrophilic iodine-iodine substitution.

Upon increase of the initial ratio of phenol/I^- the amount of triiodophenol formed as well as the ratio between the amounts of 2,4- and 2,6-diiodophenol decreases. This is in agreement with the above due to the fact that 2,4,6-triiodophenol was formed from 2,6-diiodophenol but not from 2,4-diiodophenol when alternative substrates, *e.g.* phenol were available. Since 2,6-diiodophenol only gave rise to 2,4,6-triiodophenol, the ratio 2,4-diiodophenol/2,6-diiodophenol increase, when the triiodophenol content increases.

Also of interest is that enzymatically controlled iodination of phenol in excess of hydrogen peroxide (compared to the conc. of iodide) causes the

formation of 2-iodophenol dimers, whereas 4-iodophenol apparently is not a subject to dimerization. The 2-iodophenols are probably linked through the other *ortho*-carbons. Danner et al.[4] demonstrated the enzymatically catalyzed dimerisation of phenol through the *ortho*-carbons.

It is interesting to note that the production of 2-iodophenol apparently is strongly favourized relative to 4-iodophenol. For the enzymatically controlled iodination we found a 2:3 ratio equal to ca. 6:1, corresponding to a 86% yield of 2. The direct iodination, using elemental iodine afforded an even higher 2:3 ratio, as 92% of 2 was found. However, bromination of phenol on applying elemental bromine leads to the formation of only 23% of 2-bromophenol, in agrement with recent results reported by Tee et al.,[5] who found a preference for para substitution at pH 4. In Table 2 some *ortho/para* ratios for halogenation of phenol in aqueous solutions are listed.

Table 2. *Ortho/para* ratios of the products formed, halogenating phenol

Reaction	pH	*ortho/para*	
Phenol, Bromine	pH 5	0.30	This work
Phenol, Iodine	pH 5	13.3	
Phenol, NaOCl	pH 4	0.64	(Ogata et al. 1989)
Phenol, NaOCl	pH 7	1.8	
Phenol, NaOCl	pH 8.8	2.8	
Phenol, NaOCl	pH 10	4.3	

From Table 2 it can be seen that at pH 5 the *ortho*-position is strongly favoured for iodination, while in the case of bromination apparently the para-position is favoured. At alkaline pH, Tee et al.[5] suggested that the

ortho/para ratio changed towards increasing values when brominating phenol. However, they applied equal amounts of phenol and bromine. Thus, poly-brominated phenols were formed. This may distort the *ortho/para* ratio by possible preferential consumption of *ortho*-bromophenol.[5]

Based on theoretical considerations, Ogata et al.[6] suggested that the *para*-position both in phenol and in the phenoxide ion as well as in anisole should be favoured for electrophilic substitution relative to the *ortho*-position. In their study of phenol chlorination, however, they concluded that some of the calculations support a mechanism, involving the formation of PhOCl, which subsequently may rearrange into *ortho*-chlorophenol. The mechanism is further supported by the increase of the *ortho/para* ratio, which increases with increasing pH (Table 2) and that chlorination of anisole leads to the expected *ortho/para* ratio based on the theoretical calculations Ogata et al.[6] An analogous mechanism may be involved in the iodination of phenol.

3.2. Iodination by iodide mediated by chloramine-T

Chloramine-T mediated iodination of phenol in acetatebuffer (pH 5) leads to an *ortho/para* ratio between 0.5 and 1. However, the iodide/phenol ratio was 1 and the formation of a considerable amount of di-iodophenol was observed. It can be noted that only 2,4-diiodophenol and not 2,6-diiodophenol was observed, which strongly suggests that the di-iodophenol was formed from 4-iodophenol. Kometani et al.[7] found 96% formation of 4-iodophenol by chloramine-T mediated iodination of phenol in dimethylformamide and dimethylsulfoxide. In this context it is important to note that attempts to iodinate phenol with elemental iodide in dimethylformamide revealed an extremely slow reaction, as less than 10% of the introduced iodine had reacted within 24 h. However, the predominance of 4-iodophenol was unambiguous. These findings suggest that chloramine-T mediated iodination involves a mechanism other than

iodination by elemental iodine. It should be noted that in dimethyl formamide the iodine-iodine bond in elemental iodine is strongly polarized, however, the reaction leading to the effective iodinating species, hypoiodous acid, as in aqueous solution, obviously is absent.

When treating 2-iodophenol and 4-iodophenol, respectively, with chloramine-T in acetatebuffer (pH 5), 2-iodophenol is not detectable by means of MS after one hour, but 4-iodophenol seems to be unaffected. These findings may distort the results because selective transformation of 2-iodophenol to some unknown compounds will obviously decrease the *ortho/para* ratio. It can also be noted that chloramine-T mediated iodination of phenol in acatatebuffer leads to the formation of unknown iodine-containing compounds. These species, which were difficult to elute from the HPLC column, may possibly consist of iodinated polymeric species involving the chloramine-T.[8] By means of MS no dimers of iodophenol were found, contrary to the enzymatically controlled iodination of phenol in excess of hydrogen peroxide where dimers of 2-iodophenol were produced.

3.3. Mechanistic considerations

According to Morrison and Schonbaum[9] the peroxidase may be oxidized by H_2O_2. They suggest that the oxidized form of the enzyme will interact with I^- leading to an enzyme-iodine complex, which apparently is the electropositive iodine species, which by electrophilic attack on one of the negatively charged sites in phenol leads to the iodinated species leaving the reduced lactoperoxidase:

$$LP + H_2O_2 \rightarrow LP_{ox}$$

$$LP_{ox} + I^- \rightarrow LPI$$

$$LPI + \text{phenol} \rightarrow LP + \text{iodophenol}$$

It is well known that lactoperoxidase in the presence of iodide and hydrogen peroxide may lead to the formation of elemental iodine.[10] Thus, we have to consider an alternative reaction, in which a primary formation of elemental iodine appears crucial.

$$LPI + I^- \rightarrow LP + I_2$$

It is well established that elemental iodine in aqueous solution exists in an equilibrium with hypoiodous acid (HOI). Even at pH 5 significant amount of HOI apparently is present.[11] Thus, Niedleman and Geigert[12] suggest that the iodinating species is HOI. They concluded that the results of peroxidase mediated iodination can be explained by the operation of hypoiodous acid. To elucidate the possible involvement of HOI we compared the iodination of phenol (acetate buffer, pH 5) with aqueous elemental iodine and hypoiodous acid, respectively.

When phenol (3.3×10^{-3} mole/L) was iodinated at pH 5 by aqueous elemental iodine (3.3×10^{-4} mole/L) , only 6 - 7 % of the mono-iodophenol formed was 4-iodophenol whereas 93-94% 2-iodophenol was detected. When elemental iodine was treated with equivalent amounts of NaOH the solution turned colourless, due to the formation of HOI. After adjusting pH to 5 by acetatebuffer (the solution was still colourless), phenol was added. The product constituted of 2-iodophenol and 4-iodophenol in the same ratio as above. This strongly indicates that the same iodinating species, probably HOI, operates in both cases.

$$I_2 + H_2O \rightarrow HOI + H^+ + I^-$$

$$HOI + \text{phenol} \rightarrow \text{iodophenol} + H_2O$$

Especially it should be noted that the operation of hypoiodous acid apparently explains the surprisingly high *ortho/para* ratio observed, although it is not obvious if the formation of *ortho*-iodophenol involves an intermediary

hypoiodite structure Ph-OI, which rearranges into the iodo-phenol, or if the HOI, by hydrogen bonding structurally is located in a position favourable for *ortho*-substitution. It is in this context once more worthwhile to mention the apparent predominance for *para*-substitution of phenol by interaction with elemental iodine in dimethylformamide.

In the present study we have not been able unambiguously to elucidate if the enzymatically controlled iodination of phenol takes place by interaction of phenol and the enzyme-iodine complex or by a reaction with primary formed elemental iodine/hypoiodous acid. However, it seems most reasonable to assume that both mechanisms are operating. The pronounced predominance for *ortho*-substitution found, also in the case of the enzymatically controlled reaction, strongly suggests the engagement of elemental iodine/hypoiodous acid in the iodination reaction, as it appears less likely that an interaction between the OH-moiety in the phenol and the enzyme-iodine complex should lead to the high 2:3 ratio observed. On the other hand, the tendency towards a decreased 2:3 ratio in the enzymatically controlled iodination relative to that observed for the direct iodination applying elemental iodine suggests at least some participation of a reaction between phenol and the enzyme-iodine complex. In an attempt to rationalize the above findings, we suggest that as long as iodide is present in the reaction mixture in reasonably high concentrations the latter will act as substrate for the enzyme-iodine complex leading to the formation of elemental iodine, and, hence, hypoiodous acid, which consecutively reacts with phenol. However, upon decreasing the iodide concentration during the reaction, phenol constitutes as an alternative substrate for the enzyme-iodine complex, a reaction which apparently leads to an enhanced *para*-substitution. Thus, iodination of phenol (10^{-4} M) applying iodide concentrations equal to 10^{-5}, 2.5×10^{-6}, and 10^{-6} M, respectively, lead to formation of 14.4, 18.4 and 20.6% 4-iodophenol, respectively.

4. CONCLUDING REMARKS

In 1984 Whitehead[13] stated that the typical iodine content in soils is 0.5 - 20 mg/kg corresponding to 4×10^{-6} to 1.6×10^{-4} M/kg. Obviously the here applied iodide concentrations at 1×10^{-5} to 1×10^{-4} M agree well with those found in the environment. It can be mentioned that concentrations in rain, as source for soil water are about three orders of magnitude lower.[13] As no iodophenols in detectable amounts in the present study were formed in the absence of enzyme, and since we observed that close to 90% of iodide is consumed when enzyme was available, it seems obvious that even small amounts of enzyme will increase the reaction rate dramatically. The present study has demonstrated that phenol even in rather low concentrations easily can be iodinated by I^- in the presence of lactoperoxidase and of H_2O_2 and that both mono-, di-, and tri-iodinated derivates are formed.

Taking into account that extracellular peroxidases as well as H_2O_2 are available in most soils and humic acids contain approximatly 20% phenolic, moieties[14] the results in the present work do support the hypothesis of enzymatically controlled iodination of humic substances in soil and further investigations on other model molecules as well as on humic acids of different origin are in progress.

Finally, it should be mentioned that in the case of enzymatically controlled iodination of phenol the hydrogen peroxide/iodide ratio appears to play a crucial role in determining the eventual yields of 2-iodo- and 4-iodophenol, respectively. Thus, whenever the hydrogen peroxide concentration exceeds that of the iodide ions other peroxidation reactions may operate. In the present case it was demonstrated that an excess of hydrogen peroxide relative to iodide lead to a consecutive peroxidase controlled reaction yielding significant amounts of a 2-iodophenol dimer in agreement with previous results demonstrating peroxidase induced polymerizations of phenols.[4,15] It is in this context interesting to note that 4-iodophenol apparently is not

affected by lactoperoxidase/hydrogen peroxide.

Obviously, the halogenation of phenol is not as simple as could be expected. Further elucidation of the interaction of different halogenating species with phenol is necessary if the mechanisms involved are to be described in detail.

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APPENDIX 5

Presented at the International Symposium on Humic substances In the Terrestrial and aquatic environment, Linköping, august 1989, in press.

IODINATED HUMIC ACIDS

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ABSTRACT

Humic acids are iodinated by elemental iodine and, if the iodine is present as iodide, by peroxidase mediated reactions. It is demonstrated that iodination of humic acids leads to a product with a uniform distribution of iodine. It could not be unambiguously verified whether the enzymatically mediated iodination is a direct reaction between a peroxidase-iodine complex and the humic acid molecule or a two-step reaction in which the enzyme creates elemental iodine, which consecutively reacts with the humic acid. Based on a simple model of the reaction between sites in the humic acid available for iodination and the electrophilic iodinating species, it was concluded that the reaction should be described as an equilibrium, the logarithmic equilibrium constant being approximately 4. The number of sites available for iodination was in the humic acid studies determined to be approximately $4x10^{-4}$ per gram humic acid. The different parameters influencing the enzymatically controlled iodination of humic acids are discussed.

INTRODUCTION

During the past two decades, investigations of the migration of stable radionuclides have drawn attention to the presence of organo iodine compounds in the terrestrial environment [1]. Strong evidence of microbial formation of organic iodine compounds has been presented [2-4], whereas direct reactions between inorganic iodide and soil organic matter have been considered less plausible.

In a recent study [5] we demonstrated the enzymatically controlled iodination of phenol, using iodide/hydrogen-peroxide/lactoperoxidase as the iodinating reagent. Owing to the polyphenolic nature of humic acids, incorporation of iodine into humic substances by peroxidase mediated reactions appears reasonable. The present paper summarizes our studies on iodination of commercially available humic acids (Aldrich) using lactoperoxidase (SIGMA)/hydrogen peroxide as the enzymatic catalyst.

METHODS AND MATERIALS

Chemicals

Humic acid (Aldrich), H_2O_2 (J.T. Baker Chemicals), NaI (Merck p.a.), ^{131}I as NaI, Lactoperoxidase (EC.1.11.1.7.) (Sigma L-2005), Acetate buffer pH 5, NaOH (Merck p.a.), $NaHSO_3$ (J.T. Baker Chemicals) and HCl (FERAC zur analyse).

Verification of the existence of iodinated humic acids

In a total volume of 5 ml (acetate buffer pH 5), iodide ($5x10^{-5}$ M) (spiked with $^{131}I^-$) and humic acid (0.1 g/L) were mixed. The reaction was initiated by addition

of the appropriate enzyme (appr. 5 units) and hydrogen peroxide ($2x10^{-4}$ M). The reactions were allowed to proceed for 30 min. The reaction mixtures were analyzed directly by liquid chromatography (column: 250x4.6 mm Ultrahydrogel™, eluent: 0.1 M sodium acetate, pH being adjusted to 9.6 by sodium hydroxide, flow rate: 0.6 mL/min.).

Incorporation of iodine in humic acids

The amount of iodine incorporated in the humic acid was determined as function of the initial concentrations of iodide, enzyme, humic acid and hydrogen peroxide, respectively. The effect of variation in reaction time was studied. The general procedure was as follows:

In a total volume of 5 ml (acetate buffer pH 5), iodide (spiked with $^{131}I^-$) and humic acid were mixed. The reaction was initiated by addition of the appropriate enzyme and hydrogen peroxide. After the allowed reaction time (mostly 10 min) 1 ml of $NaHSO_3$ (0.1 M) was added to terminate the reaction. The reaction mixture was transferred to a clean vessel in order to eliminate iodine sorbed to the internal surface of the original reaction vessel and 1 ml of HCL (12 M) was added. The reaction mixture was allowed to rest for 5 min. and subsequently centrifugated for 25 min. (3000 rpm). 4 ml of the supernatant were subjected to gamma-counting. 2 ml of NaOH solution (2M) was added to the vessel containing the precipitated humic acid and the rest solution to dissolve the precipitated material, the solution subsequently being subjected to gamma-counting. The distribution of iodine in humic acid and in the solution was then calculated based on the mutual counting rates.

The inhibiting effect of humic acid on the enzyme function was investigated allowing lactoperoxidase and humic acid to be in contact before initiating the iodination reaction by adding hydrogen peroxide.

The equilibrium was investigated allowing the reaction to proceed for 20 hours applying varying initial concentrations of iodide in the range from $2x10^{-5}$ to $2x10^{-4}$ M. The distribution between bound and free iodine was carried out as described above.

IODINATION OF HUMIC ACIDS

In the presence of lactoperoxidase (LP) and hydrogen peroxide, humic acids (HA), dissolved in an acetate buffer (pH 5), reacted readily with iodide. Analysis of the HA by liquid chromatography (Fig. 1) demonstrated that iodine was incorporated into all molecular weight fractions of the HA. Hence, the reaction product can be described as iodinated humic acids.

The fact that the iodine incorporation took place in all molecular weight fractions of the HA indicated that humic acids are composed of certain "basic units", the number of which determines the molecular weight of the single humic acid molecule. Model experiments with phenols indicated that phenolic sites would be available for iodination [5]. However, all types of phenolic unit are not readily available for iodination. This was demonstrated in a series of model experiments with polysubstituted phenolic structures believed to reflect at least some of the phenolic moieties in HA [6-8].

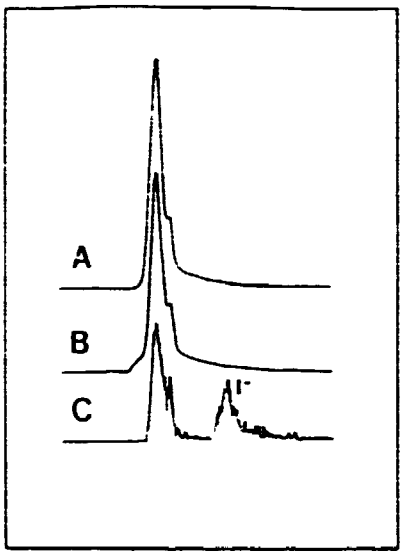

Figure 1. Chromatographic trace of A: humic acid prior to iodination (UV-detection), B: humic acid after iodination (UV-detection), C: humic acid after iodination (^{131}I detection). Initial concentrations: humic acid, 0.1 g/L; iodide, 3E-5M; hydrogen peroxide, 2E-4 M; enzyme, 10 µg/mL. Reaction time: 20 min.

It is important to note that HA may be iodinated by elemental iodine. Using liquid chromatography (not shown) it was demonstrated that this reaction leads to the same molecular weight distribution of iodine as the above described enzymatically controlled iodination. However, under the conditions normally prevailing in the environment, iodine will predominantly exist as anionic iodide [1]. Furthermore, in the concentration range investigated ($[I^-]$ 2.5×10^{-4} M) hydrogen peroxide ($[H_2O_2]$ 2×10^{-4} M) was not able to convert iodide into elemental iodine in the course of the reaction times studied. This shows that the incorporation of iodine into HA cannot be attributed to a simple chemical oxidation. The exact mechanism of the enzymatically controlled iodination is not known. The formation of iodinated HA can be a result of a reaction directly involving a lactoperoxidase-iodine complex or a reaction of elemental iodine generated during a primary enzyme activity. Possibly a composite reaction should be considered, where the formation of elemental iodine initially operates, whereas the direct engagement of an enzyme-iodine complex prevails for low iodide concentrations, e.g. towards the end of the reaction.

In the following the influence of the different parameters on the iodination reaction shall be discussed.

Influence of Humic Acid Concentration

In the presence of excess iodide, the amount of incorporated iodine was expected to increase linearly with the humic acid concentration. However, to our surprise, the observed incorporation increased less than theoretically predicted. More precisely, the amount of incorporated iodine increased only from $8.6x10^{-6}$ to $3.5x10^{-5}$ mol/L when the initial HA concentration increased from 0.05 and 0.4 g/L. This tendency was even more pronounced when the initial humic acid concentration was increased to 1.0 g/L. The corresponding iodine incorporation was then found to be $3.4x10^{-5}$ mol/L. All experiments were carried out with initial concentrations of iodide hydrogen peroxide and lactoperoxidase equal to $1x10^{-4}$ M, $2x10^{-4}$ M and 10 µg/mL, respectively.

A reasonable explanation of the apparently decreasing incorporation of iodine with increasing humic acid concentration seems to be a deactivation of the enzyme function due to complexation between the humic acid and the enzyme [9]. In order to obtain support for this hypothesis, we carried out a series of experiments in which the humic acid and the enzyme was contacted for 1 h prior to the addition of iodide and hydrogen peroxide. Compared to the above experiments, a significant decrease in enzyme activity was observed in all cases. For initial concentrations of humic acids equal to 0.1, 0.2 and 0.4 g/L the decrease in enzyme activity was determined to 7, 11 and 21%, respectively. We concluded that the concentration of "active enzyme" plays an important role.

Influence of Enzyme/Hydrogen Peroxide Concentrations

The incorporation of iodine into HA increased with increasing enzyme concentrations. For initial iodide, hydrogen peroxide and HA concentrations equal to $3x10^{-5}$ M, $2x10^{-4}$ M and 0.1 g/L, the amount of incorporated iodine was found to be 1.6, 3.5, 7.1 and 8.2 $x10^{-5}$ M/gHA for enzyme concentrations equal to 2, 4, 10 and 20 µg/mL, respectively. However, further experiments showed that the hydrogen peroxide concentration also plays a dominant role in determining the enzyme activity. Increasing the initial hydrogen peroxide concentration from $5x10^{-5}$ to $8x10^{-4}$ M caused a 60% decrease in iodine incorporation. A priori, one would have expected an increased enzyme activity by increasing the hydrogen peroxide concentration, due to an increased rate of formation for the oxidized form of the peroxidase (LP_{ox}). However, increasing the hydrogen peroxide concentration above a certain limit apparently caused a deactivation, or possibly destruction, of the enzyme function.

The results are summarized in Fig.'s 2 and 3.

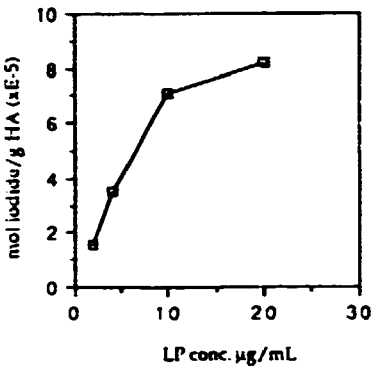

Figure 2. Iodine incorporation as function of enzyme concentration (Initial concentrations: iodide: 3E-5 mol/L, hydrogen peroxide: 2E-4 mol/L, humic acid: 0.1 g/L)

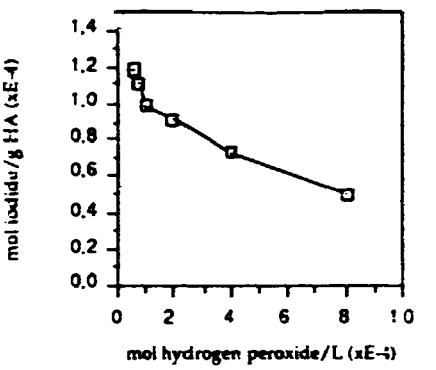

Figure 3. Iodine incorporation as function of hydrogen peroxide concentration (Initial concentrations: iodide: 5E-5 mol/L, enzyme: 10µg/mL, humic acid: 0.1 g/L)

Influence of Iodide Concentration

As expected, the amount of iodine incorporated into the HA varied with the initial iodide concentration. In Fig.'s 4 and 5 the amount of iodine consumed by the HA is related to the initial iodide concentration. The curves in Fig. 4 show a pronounced tendency to levelling-off phenomena. This effect is further elucidated by Fig. 5, displaying the percentage of the initial iodide being incorporated in the humic acids. The curves depicted in Fig 5 correspond to the first derivative of the corresponding curves displayed in Fig. 4. Optimal iodide consumption apparently takes place for initial iodide concentrations around 2.5×10^{-5} mol/L. The mechanistic aspects of these findings are outlined below.

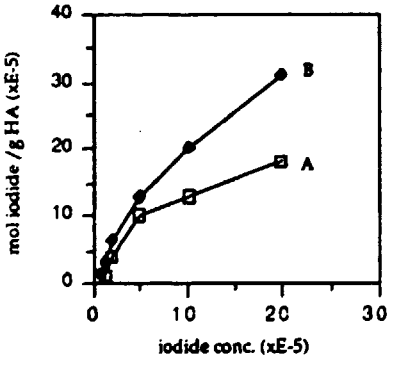

Figure 4. Iodine incorporation as a function of initial iodide concentration (initial concentrations: hydrogen peroxide: 2E-4 mol/L, enzyme: 10 µg/mL, humic acid: 0.1 g/L). Reaction time: A: 10 min, B: 4h.

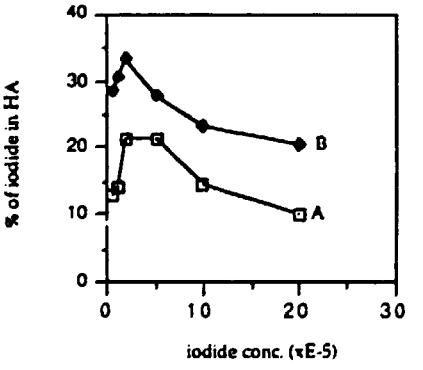

Figure 5. Percentage of iodine incorporated as a function of initial iodide concentration (Initial concentrations: hydrogen peroxide: 2E-4 mol/L, enzyme: 10 µg/mL, humic acid: 0.1 g/L). Reaction time: A: 10 min, B: 4h.

Influence of Reaction Time

As already noted from the above figures (Fig.'s 4 and 5) the incorporation of iodine into humic acids increased with the contact time. However, in order to obtain further insights in the kinetics of the humic acid iodination reaction, we studied the reaction over a period of time up to 24 hours. In Fig. 6 the time profiles for initial iodide concentrations equal to $5x10^{-5}$ and 10^{-4} M are visualized. It is immediately seen that the maximum incorporation of iodine is reached rather rapidly, and at approx. 4 h the final level of iodine consumption has virtually been reached.

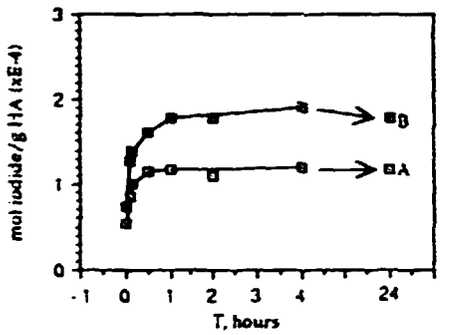

Figure 6. Incorporation of iodine into humic acid as a function of reaction time for initial iodide concentrations equal to A: 5E-5 mol/L and B: 1E-4 mol/L.

The sum of the above findings has lead us to the following mechanistic considerations concerning the iodination of humic acids.

MECHANISM OF HUMIC ACID IODINATION

It is interesting to note that a prolonged reaction time did not lead to more than approximately 35-40% consumption of the initially applied iodide (cf. Fig. 5). This is in contrast to previous studies on the iodination of phenol, in which a complete iodide consumption was obtained under reaction conditions comparable to those of the present study. The levelling-off phenomena observed for increasing initial iodide concentrations (Fig. 4) and the time profiles in Fig. 6 strongly suggest that the iodination of HA should be considered as an equilibrium reaction.

Owing to the above described findings, we have to consider a wide range of reactions, which jointly may be responsible for the iodination of humic acids. These reactions comprise oxidation of LP to LP_{ox} by hydrogen peroxide, reaction of LP_{ox} with I^- leading to the iodinating enzyme- iodine complex LP-I and reaction of the latter with iodide and/or humic acids to form elemental iodine and/or iodinated humic acids, respectively. As previously stated it has not been possible to elucidate to what extent the two latter reactions operate.

In an attempt to explain the above results, we propose a simple equilibrium model taking into account only the concentration of sites available for iodination, [S], the concentration of the iodinating species, ["I"] and the concentration of iodinated sites, [SI]. The initial concentration of the iodinating species are put equal to the initial iodide concentration, i.e. $["I"]_o = [I^-]_o$.

$$S + "I" \rightleftharpoons SI$$

This equilibrium system gives rise to the following expression

$$\frac{[SI]}{[S]["I"]} = \beta$$

which can be rewritten into

$$\frac{[I^-]_o}{1 + Q} = q\frac{[HA]}{Q} - \frac{1}{\beta}$$

where Q is equal to the ratio between the concentrations of incorporated iodine and free iodide in solution and q is the number of sites available for iodination per gram HA. β is the equilibrium constant.

Obviously, a plot of $[I^-]_o/(1 + Q)$ vs. [HA]/Q should give a straight line, where the slope would be the number of sites available for iodination per gram humic acid, q. The equilibrium constant may be derived from the intercept $-1/\beta$.

The applicability of this rather simple model for the enzymatically controlled iodination of humic acids seems verified by the plot displayed in Fig. 7. A linear relationship between $[I^-]_o/(1 + Q)$ and [HA]/Q was obtained for initial iodide concentrations $[I^-]_o$ from 2×10^{-5} to 2×10^{-4} M. Based on a least-square procedure, the number of sites available for iodination was found to be $q = 4.28\pm0.22\times10^{-4}$ sites per gram HA. The equilibrium constant was estimated to $\beta = 1.32\pm0.15\times10^{4}$.

A priori, the number of sites available for iodination may seem rather low. However, looking at proposed models for humic acid structures, it appears obvious that only few "free" aromatic hydrogens, i.e. potentially available sites, are present [10]. Furthermore, taking into account that a significant number of the potentially available sites in fact must be considered as non-available, probably due to an enzyme-inhibiting effect [7], the obtained number appears reasonable.

Finally it can be mentioned that a logarithmic equilibrium constant in the range of 4 is in fully agreement with an observed rather slow release of iodide from isolated iodinated humic acids [11].

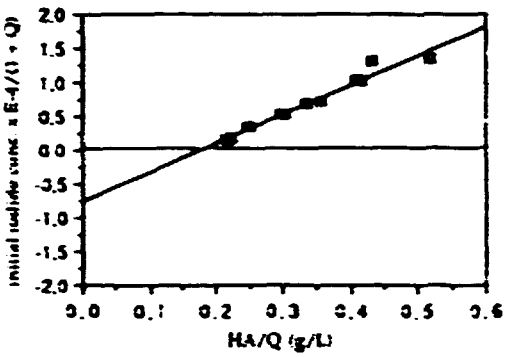

Figure 7. Plot of initial iodide concentration/(1 +Q) as function of HA/Q

CONCLUSION

It has been demonstrated that iodinated humic acids can be obtained either by a direct reaction between humic acids and elemental iodine or with iodide in the presence of lactoperoxidase and hydrogen peroxide. The reaction, which is regarded as an electrophilic aromatic substitution, can, at least for a certain range of concentrations of reactants, satisfactorily be described as a simple equilibrium between humic acids and the electropositive iodinating species.

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APPENDIX 6

Presented at the Second International Conference on Chemistry and Migration Behavior of Actinides and Fission Products in the Geosphere, Monterey, november 1989, in press.

ENZYMATICALLY CONTROLLED IODINATION REACTIONS IN THE TERRESTRIAL ENVIRONMENT

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ABSTRACT

Humic acids are iodinated either by elemental iodine/hypoiodous acid or by iodide in the presence of enzymes of the peroxidase group and hydrogen peroxide. The resulting iodinated humic acids show a uniform distribution of the iodine independent of the single molecular weight fractions. It is demonstrated that the enzymatically controlled iodination reactions can be rationalized as applying to a rather simple equilibrium model taking only the concentrations of the sites available for iodination and the initial iodide concentrations into account together with the concentration of the iodinated sites. Deiodination experiments suggest that three different types of sites available for iodination are present, distinguishable due to the deiodination mechanisms. The possible influence of the findings on the migration behaviour of radioiodide in the terrestrial environment is discussed.

INTRODUCTION

For many years major attention to the environmental cycle of iodine has been paid due to its status as essential element to man. In recent years, after the introduction of nuclear power as an energy source, the environmental cycle of iodine has received renewed interest due to the possible release of the longlived radioactive ^{129}I isotope, e.g. during reprocessing [1]. Man concentrates iodine in the thyroid gland. Hence, high ^{129}I levels in the environment, and subsequently high levels in the human thyroid gland may cause servere damages, i.e. development of cancer.

The geochemistry of iodine, especially factors influencing the migration behavior of iodine with perculating rainwater and groundwater is of major interest in order to predict the behaviour of radioiodine in the environment. The iodine content in soil seems to be positively correlated with the organic content, and organic material apparently exhibits a pronounced tendency to retain iodine [2-4]. In the following we shall discuss mechanisms possibly being responsible for the retention of iodine in soil.

Cooksey et al. [5] demonstrated that different well established breakdown products from humic substances (e.g. resorcinol) exhibited antithyroid effect to rats when added to drinking water in low concentrations. In the thyroid gland iodine (as I^-) is incorporated in the amino acid tyrosin by an enzymatically controlled process. Thus, the antithyroid effect could be caused by blocking the enzyme. However, it appears possible, due to the structural similarities between tyrosin and the "antithyroid compounds" that it is a question of competitive inhibition, i.e. that the inhibiting molecule is iodinated instead of tyrosin. The results from Fawcett and Kirkwood [6] who find iodoresorcinol after treating thin slices of rat thyroid with resorcinol seem to support this hypothesis.

Iodination of tyrosin in the thyroid gland is catalyzed by an enzyme of the peroxidase group, "thyroid peroxidase", in the presence of hydrogen

peroxide. Thus, if substances, structurally related to humic material are iodinated enzymatically in the thyroid gland, it seems possible that related reactions may occur in soil, i.e. iodination of humic acids.

Behrens [7-9] examined soil and soil water and found that I^- was incorporated in different organic compounds. It was suggested that the reactions were catalyzed by extracellular enzymes, possibly peroxidases. It should in this context be noted that extracellular peroxidases are widely distributed in soils [10]. Additionally, hydrogen peroxide is in general available in ground waters as well [9].

Humic substances contain on an average basis 20% phenolic moieties [11]. Thus, apparently the factors necessary for an enzymatically controlled iodination of humics in soil are present.

It is well known that enzymes of the peroxidase group under certain conditions are able to promote halogenation reactions [12], e.g. iodinations, either by a direct reaction between an enzyme-iodine complex and the substrate or by primary production of elemental iodine, which consecutively operates as the iodinating species.

In a previous study [13] we investigated the iodination of phenol. It was concluded that phenol could be iodinated either by elemental iodine/hypoiodous acid or enzymatically controlled, using LP in the presence of iodide and hydrogen peroxide, in concentrations as low as 10^{-4} M for phenol and 10^{-6} M for iodide, leading to a mixture of mono-, di and triiodophenols, at pH 5. Our results suggested that in the case of enzymatically controlled iodination of phenol two possible reaction pathways operates. Whereas the primary attack of elemental iodine on phenol preferentially takes place in the 2 position the interaction between the LP-iodine complex and phenol apparently leads to enhanced substitution in the 4-position.

$$\text{Enzyme} + H_2O_2 \rightarrow \text{Enzyme-ox}$$

$$\text{Enzyme-ox} + I^- \rightarrow \text{Enzyme-ox-I}$$

$$\text{Enzyme-ox-I} + \text{Substrate} \rightarrow \text{Substrate-I} + \text{Enzyme}$$

or

$$\text{Enzyme-ox-I} + I^- \rightarrow I_2 + \text{Enzyme}$$

$$I_2 + H_2O \rightarrow HOI + H^+ + I^-$$

$$\text{Substrate} + HOI \rightarrow \text{Substrate-I} + H_2O$$

Scheme 1

The present paper describes our attempts to study the enzymatically controlled iodination of a series of phenolic reference structures as well as humic acids, in order to elucidate the potential of soil organic matter as a possible sink for radioiodine in the terrestrial environment.

The major part of our investigations has been carried out using lactoperoxidase (LP) as the enzyme, in accordance with the suggestion of Behrens [7]. However, since enzymes produced by plants and fungi appear as the more likely in the ground water system also a series of studies applying horse-radish peroxidase (HRP) and chloroperoxidase (CP), the latter being produced from the fungus Caldariomyces fumago, has been carried out.

EXPERIMENTAL

Chemicals: Humic acid (Aldrich), H_2O_2 (J.T. Baker Chemicals), NaI (Merck p.a.), ^{131}I as NaI, Lactoperoxidase (EC.1.11.1.7.) (Sigma L-2005), Horseradish peroxidase (EC.1.11.1.7.) (Sigma P-8125), Chloroperoxidase (EC.1.11.1.10.) (Sigma C-0278), Acetate buffer pH 5, Phosphat buffer pH 3, NaOH (Merck p.a.), $NaHSO_3$ (J.T. Baker Chemicals) and HCl (FERAC zur analyse). Phenol (p.a.), o-phthalic acid (für chromatographie), resorcinol (p.a.) and orcinol was obtained from Merck, 3,4-dihydroxy benzoic acid (97-98%) and 3,5-dihydroxy

benzoic acid (97%) and m-phthalic acid was obtained from Aldrich and phloroglucinol (p.a.) from Fluka. All reference structure were used without further purification.

Iodination of Reference Structures:

Enzymatically controlled iodination: In a total volume of 500 mL (acetate buffer, pH 5) the single reference structures including phenol (10^{-4} M) were mixed with iodide (10^{-4} M), hydrogen peroxide (10^{-4} M) and LP (2 µg/mL). The reactions were allowed to proceed for 10 min (stirring). 1mL sodium thiosulphate (0.1 M) was added to reduce any elemental iodine formed and the reaction mixture was extracted with diethyl ether (3x100 mL). The combined ether phase was dried (sodium sulphate) and evaporated to dryness. The products were analyzed by Field Ionization/Field Desorption Mass Spectrometry.

Iodination of reference structure by elemental iodine/hypoiodous acid: In a total volume of 500 mL (acetate buffer, pH 5) the single reference structures including phenol (0.1 M) were mixed with 13 g of elemental iodine. The reaction mixtures were stirred for 24 hours. 10 mL sodium thiosulphate (0.05M) were added and the reaction mixtures were extracted with diethyl ether (5x100 mL). The combined ether phase was dried (sodium sulphate) and evaporated to dryness. The products were analyzed by Field Ionization/Field Desorption Mass Spectrometry.

Influence of reference structures on the enzymatically controlled iodination of phenol: In a total volume of 5 mL (acetate buffer, pH 5) phenol (10^{-4} M) was mixed with the single reference structures (10^{-4} M), iodide (10^{-4} M), spiked with ^{131}I, hydrogen peroxide (10^{-4} M) and LP (2µg/mL). The reactions were allowed to proceed for 10 min. The reaction mixtures were subjected directly to HPLC analysis (column: 250x4.6 mm Nucleosil C8 (5µ), eluent: methanol/water (62/38), flow rate: 0.6 mL/min) without further purification.

Enzymatically Controlled Iodination of Humic Acids:

Verification of the existence of iodinated humic acids: In a total volume of 5 ml (acetate buffer pH 5), iodide ($5x10^{-5}$ M) (spiked with $^{131}I^-$) and humic acid (0.1 g/L) were mixed. The reaction was initiated by addition of the appropriate enzyme (appr. 5 units) and hydrogen peroxide ($2x10^{-4}$ M). The reactions were allowed to proceed for 30 min. The reaction mixtures were analyzed directly by liquid chromatography (column: 250x4.6 mm Ultrahydrogen™, eluent: 0.1 M sodium acetate, pH being adjusted to 9.6 by sodium hydroxide, flow rate: 0.6 mL/min.).

Incorporation of iodine in humic acids: In a total volume of 5 ml (acetate buffer pH 5), iodide (spiked with $^{131}I^-$) and humic acid were mixed. The reaction was initiated by addition of the appropriate enzyme and hydrogen peroxide. After the allowed reaction time 1 ml of $NaHSO_3$ (0.1 M) was added to terminate the reaction. The reaction mixture was transferred to a clean vessel in order to eliminate iodine sorbed to the internal surface of the original reaction vessel and 1 ml of HCL (12 M) was added. The reaction mixture was allowed to rest for 5 min. and subsequently centrifugated for 25 min. (3000 rpm). 4 ml of the supernatant were subjected to gamma-counting. 2 ml of NaOH solution (2M) was added to the vessel containing the precipitated humic acid and the rest solution to dissolve the precipitated material, the solution subsequently being subjected to gamma-counting. The distribution of iodine in humic acid and in the solution was then calculated based on the mutual counting rates.

The reversability of the enzymatically controlled iodination of humic acid was investigated by isolation of iodinated humic acid by acid precipitation (HCl) followed by dissolution of the precipitated material by sodium acetate and acetatebuffer, pH being ajusted to 5. The dissolved humic acid were subsequently subjected to deiodination by addition of iodide, H_2O_2, and

enzyme and/or elemental iodine in varying combinations. The distribution between bound and free iodine was carried out as described above.

The equilibrium was investigated allowing the reaction to proceed for 20 hours applying varying initial concentrations of iodide in the range from $2x10^{-5}$ to $2x10^{-4}$ M.The distribution between bound and free iodine was carried out as described above.

RESULTS AND DISCUSSION

Iodination of Reference Structures

In order to elucidate the iodination of phenolic structures we investigated a series of substrates comprising phenol as well as eight structures generally accepted as decomposition products from humic substances including resorcinol, phloroglucinol, orcinol, 3,4-dihydroxy bonzoic acid, 3,5-dihydroxy benzoic acid, o-phthalic acid and m-phthalic acid. These structures exhibit a variety of positions in the aromatic core, which a priori can be regarded as available for iodination. However, it could be expected, due to steric as well as electronic effects that iodination would take place to quite different extents and with different rates, respectively. Furthermore, the possiblity of an interaction between the substrates and the enzyme, possibly affecting the enzyme function, might play an important role.

In 1985 Cooksey et al. [5] described how the above mentioned phenolic compounds caused diminished incorporation of ^{125}I in thin slices of hog thyroid gland and disturbed the function of thyroid glands in rats. It was concluded that the function of the thyroid peroxidase, responsible for the iodination of the thyroid hormone, was inhibited by these compounds.

The observed disturbed function of the thyroid gland can be ascribed to inhibition of the enzyme thyroid peroxidase. Thus the apparent inhibition

could a priori either be due to competitive iodination reactions involving the so-called "antithyroid" aromatic compounds at the experse of the thyroid hormone or by structural blocking of the enzyme activity.

To elucidate whether the above mentioned aromatic "antithyroid" compounds possibly would influence the activity of the lactoperoxidase, the enzymatically controlled iodination of phenol were carried out in the presence of the above mentioned aromatic compounds. The results demonstrated the same trend as noted by Cooksey et al. [5]. Resorcinol, phloroglucinol, orcinol and 3,5-dihydroxy benzoic acid inhibited the iodination of phenol completely, while 3,4-dihydroxy benzoic acid, o-phthalic acid and m-phthalic acid did not influence the iodination of phenol (in concentrations of 10^{-4} M). In addition it should be noted that in the case of orcinol the latter was iodinated to a minor extent.

It was demonstrated by application of mass spectrometric analysis that elemental iodine/hypoiodous acid is able to iodinate resorcinol, phloroglucinol, orcinol and 3,5-dihydroxy benzoic acid and to a very minor extent (trace amounts only) 3,4-dihydroxy benzoic acid, however, not o-phthalic acid and m-phthalic acid. In the case of resorcinol and phloroglucinol both mono-, di- and tri iodinated species was observed, whereas orcinol gave rise to only mono- and diiodinated isomers. In the case of 3,5-dihydroxy benzoic acid 4-iodo-3,5-dihydroxy benzoic acid was the only product observed. Thus, the above lack of enzymatically controlled iodination of the "antithyroid" compounds cannot be explained by a lacking reactivity towards iodination in general. On this background, it seems resonable to conclude that the fact that the "antithyroid" compounds in generel was not subject to enzymatically controlled iodination reflects a blocking of the enzyme by these compounds. However the mechanism is not quite obvious as some iodination of orcinol seems to prevail . On the other hand, a support for competetive iodinations can be mentioned, as Fawcett and Kirkwood [6] find iodoresorcinol when treating thin slices of rat thyroid with resorcinol.

On this background reaction mixtures obtained from the phenolic compounds treated with iodide, hydrogen peroxide and lactoperoxidase in acetatebuffer pH 5 were analyzed using mass spectrometry to elucidate whether iodinated species, which was not detectable using HPLC was formed. It was demonstrated that only mono-iodo orcinol, mono-, di- and tri-iodo phenol was formed and to a minor degree 4-iodo-3,5-dihydroxy benzoic acid, the latter being confirmed by means of HPLC.

Iodination of Humic Substances

Since humic substancescontain a wide variety of phenolic substructures [11], it appears most probable that some of these structures will be available for iodination.

Based on liquid chromatographic studies we have unambiguously demonstrated that humic acids react readily with iodide in the presence of an enzyme of the peroxidase group and hydrogen peroxide. In Figure 1 the chromatographic traces of the humic acids before and after iodination (UV detection) and after iodination (^{131}I detection) are shown. It can be noted that virtually identical results are obtained using LP, HRP and CP, respectively, as only minor differencies in the ratio between bound and free iodine can be noted, a priori reflecting the differencies in enzyme effectiveness under the given conditions (pH 5). The iodination reaction using CP was studied at pH 3 as well, under which condition it is known that the enzyme exhibits its maximum effectiveness. However, we noted that a decreased amount of iodine was incorporated in the humic acids. This result seems a priori surprising. However, the decreased solubility of the humic acid at pH 3 may in this context play a crucial role and we have not, in the frame of this study pursued this further. It is noted that the iodine incorporation apparently takes place equally in all molecular weight fractions of the humic acids.

>>>>>>>>>> Figure 1

It is important to note that humic acids may be iodinated by action of aqueous elemental iodine as well. Based on liquid chromatography (not shown) this reaction apparently leads to the same products as does the above described enzymatically controlled reaction involving iodide. Thus, an identical uniform incorporation of iodine into the humic acid moieties was noted. However, under the conditions typically prevailing under environmental conditions, iodine will predominantly exist as anionic iodide [1]. Furthermore, in the concentration range investigated, i.e. $[I^-] \leq 2.5 \times 10^{-4}$ M, hydrogen peroxide (conc. $\leq 2 \times 10^{-4}$ M) was not able to convert iodide into elemental iodine within the time frames studied. Nevertheless, in contrast to our study on the iodination of phenol [13], it could not unambiguously be concluded, whether the formation of iodinated humic acids was a result of a reaction directly involving an enzyme-iodine complex or a reaction of elemental iodine/hypoiodous acid generated during a primary enzyme activity. As in the case of phenol iodination it appears possible that a composite reaction should be considered, where both formation of elemental iodine, as well as direct engagement of an enzyme-iodine complex operates.

In a recent paper [14] we suggested to formulate the iodination of humic acids by iodide in the presence of enzyme and hydrogen peroxide as an "equilibrium" reaction, taking into account only the concentration of sites available for iodination, [S], the concentration of the iodinating species, ["I"] and the concentration of iodinated sites, [SI]. The initial concentration of the iodinating species are put equal to the initial iodide concentration, i.e. $["I"]_o = [I^-]_o$.

$$S + "I" \rightleftharpoons SI$$

This equilibrium system gives rise to the following expression

$$\frac{[SI]}{[S]["I"]} = \beta$$

which can be rewritten into

$$\frac{[I^-]_o}{1 + Q} = q\frac{[HA]}{Q} - \frac{1}{ß}$$

where Q is equal to the ratio between the concentrations of incorporated iodine and free iodide in solution and q is the number of sites available for iodination per gram humic acid. ß is the equilibrium constant.

Obviously, a plot of $[I^-]_o/(1 + Q)$ vs. [HA]/Q should give a straight line, where the slope would be the number of sites available for iodination per gram humic acid, q. The intercept equal to -1/ß leads to the equilibrium constant.

In Figure 2 $[I^-]_o/(1 + Q)$ vs. [HA]/Q plots are given for the reactions using LP, HRP and CP, respectively, as the enzyme catalyst. The initial iodide concentration was varied in the range from $2x10^{-5}$ to $2x10^{-4}$ M. The humic acid concentration was kept equal to 0.1 g/L.

>>>>>>>>>> Figure 2.

It is immediately seen that in all three cases linear correlations between $[I^-]_o/(1 + Q)$ and [HA]/Q were obtained, the correlation coefficients, r^2, being found above 0.99 in the cases of LP and CP. In the case of HRP a somewhat less significant correlation, $r^2 > 0.93$ was found. These findings strongly suggest the applicability of the above proposed simple equilibrium model to describe the enzymatically controlled iodination of humic acids. Based on a least square procedure the number of sites available for iodination as well as the equilibrium constants were calculated, the results being summarized in Table 1.

>>>>>>>>>> Table 1

The mechanisms of iodination reactions using peroxidases as the enzyme catalysts has been discussed by Morrison and co-workers [12,15,16]. They concluded that both direct action of an enzyme-iodine complex as well as a primary formation of elemental iodine/hypoiodous acid, which subsequently acts as the iodinating species have to be taken into account. The results presented in Table 1 suggests that different mechanisms to a certain extent operate in the case of LP and CP. Application of CP as the enzyme catalyst apparently leads to a somewhat diminished number of sites available for iodination and, on the other hand an increased equilibrium constant compared to the values obtained using LP. Owing to lack of knowledge concerning the actual structure of humic acids it appears not possible to pursue these differencies further. In the case of HRP it can be noted (cf. Fig. 2) that for low initial iodide concentration the results appear rather scattered. A possible explanation could be that the equilibrium situation at low iodide concentration is reached only with difficulty. Thus, it is not possible to conclude whether HRP leads to results comparable to those obtained applying LP or CP, or whether three different results are to be expected.

As a consequence of our formulation of the iodination of humic acids by iodide in the presence of enzyme and hydrogen peroxide, it is a priori to be expected that iodide will be eliminated from isolated iodinated humic acid upon dissolution. In order to study the possible deiodination process in detail we isolated small portions of iodinated humic acid labelled with ^{131}I, which subsequently were dissolved in a variety of media containing the possible reactant, i.e. enzyme, hydrogen peroxide and iodide or elemental iodine in varying mutual ratios in an acetate buffer (pH 5). These experiments have been carried out using LP as the enzyme. In Figure 3 the results of the deiodination experiments are summarized.

>>>>>>>>>> Figure 3.

It can be noted (Fig. 3) that the results of the deiodination experiments are separated in three distinguishable groups. 1° A, B, and C, where the iodinated humic acids were dissolved in acetate buffer, acetate buffer containing LP and acetate buffer containing LP and hydrogen peroxide, respectively. These three systems resulted in a release of approximately 14% iodine. 2° D and E, where the iodinated humic acids were dissolved in acetate buffer containing iodide and iodide and LP, respectively, in which cases an approximately 18% iodine release was observed. 3° F, G and H, where the iodinated humic acids were dissolved in acetate buffer containing, iodide, LP, hydrogen peroxide, elemental iodine and elemental iodine, LP, respectively. For these three systems an approximately release of iodine was found to be 40%.

These results suggest that possibly three different types of sites in the humic acids available for iodination are present. 1° sites where iodine is rather weakly bound to the humic acid structure, e.g. as π-complexes as formulated by Allinger et al. [17], 2° sites susceptible for nucleophilic iodide-iodide substitution, a reaction type known to operate e.g. in certain cases of production of iodinated radiopharmaceuticals [18], and 3° sites susceptible for a conventional electrophilic iodine-iodine substitution, a reaction which has also been observed in the case of enzymatically iodination of phenol [13]. It should be noted that the only slight increase in the iodine release originating from the proposed nucleophilic aromatic substitution compared to the pronounced increase in the case where electrophilic aromatic substitution prevail, is in agreement with the general assumption concerning the possible operation of these two types of mechanisms [19].

CONCLUSION AND OUTLOOK

It has been demonstrated that humic acids can be iodinated either by elemental iodine or iodide by action of peroxidases in the presence of hydrogen peroxide. The iodinated humic acids exhibit a uniform distribution of the iodine independent of molecular weight fractions. It appeared that the iodination reaction can be rationalized as an equilibrium reaction applying to a rather simple model, although it seems to involve different types of reactions as well as different types of sites in the humic acids.

It has not been possible unequivocally to rationalize the enzymatically controlled reactions, i.e. to elucidate to what extent the reactions are to be formulated as a direct interaction between the humic acids and enzyme-iodine complexes or as primary enzymatically controlled formation of elemental iodine, which subsequently reacts with the humic acids leading to the iodinated species.

In attempts to evaluate soil organic matter as a potential sink for radioiodine in the terrestrial environment, it seems appropriate to distinguish between soluble and solid organic matter. It appears the iodinated humic acids can be precipitated as such, however, when dissolved at least part of the iodine will be released to the liquid phase as a result of the euquilibrium reaction. Hence, we conclude that if conditions where dissolved soil organic matter is precipitated and subsequently dissolved prevail retention of iodide may be observed, the migration behaviour being controlled by an effective retention factor as recently discussed by Carlsen et al. [20]. In order to evaluate the possible release of iodine from irreversible precipitated soil organic matter further experiments have to be conducted.

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Table 1. Number of sites, q (per g HA), available for iodination and equilibrium constants, ß (L/M), as function of enzyme catalyst.

Enzyme	q	ß
LP	$(4.28\pm0.22)\times10^{-4}$	$(1.32\pm0.17)\times10^{4}$
HRP	$(4.25\pm0.50)\times10^{-4}$	$(1.56\pm0.47)\times10^{4}$
CP	$(3.48\pm0.17)\times10^{-4}$	$(2.63\pm0.40)\times10^{4}$

FIGURE CAPTIONS

Figure 1. Chromatographic trace of A: humic acid prior to iodination (UV detection), B: humic acid after iodination with HRP (UV detection) C: humic acid after iodination with HRP (^{131}I detection), D: humic acid after iodination with LP (UV detection) E: humic acid after iodination with LP (^{131}I detection), F: humic acid after iodination with CP (UV detection) G: humic acid after iodination with CP (^{131}I detection).

Figure 2. Plot of $[I^-]_o/(1 + Q)$ vs. [HA]/Q for diffenret enzyme catalysts: A: LP, B: HRP, and C: CP.

Figure 3. Graphic representation of the distribution of bound and free iodine following deiodination experiments. Media: 1° A: acetate buffer, B: acetate buffer containing LP, C: acetate buffer containing LP and hydrogen peroxide, D: acetate buffer containing iodide, E: acetate buffer containing iodide and LP, F: acetate buffer containing, iodide, LP and hydrogen peroxide, G: acetate buffer containing elemental iodine and H: acetate buffer containing elemental iodine and LP.

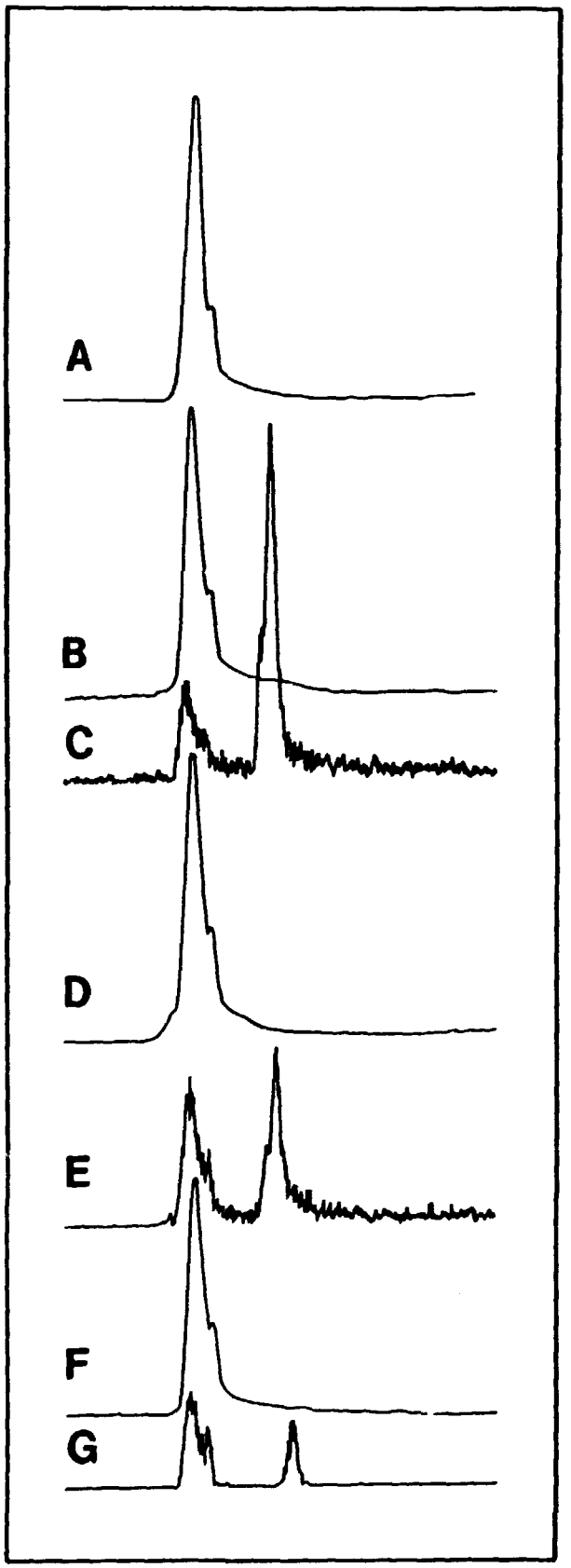


Fig. 1

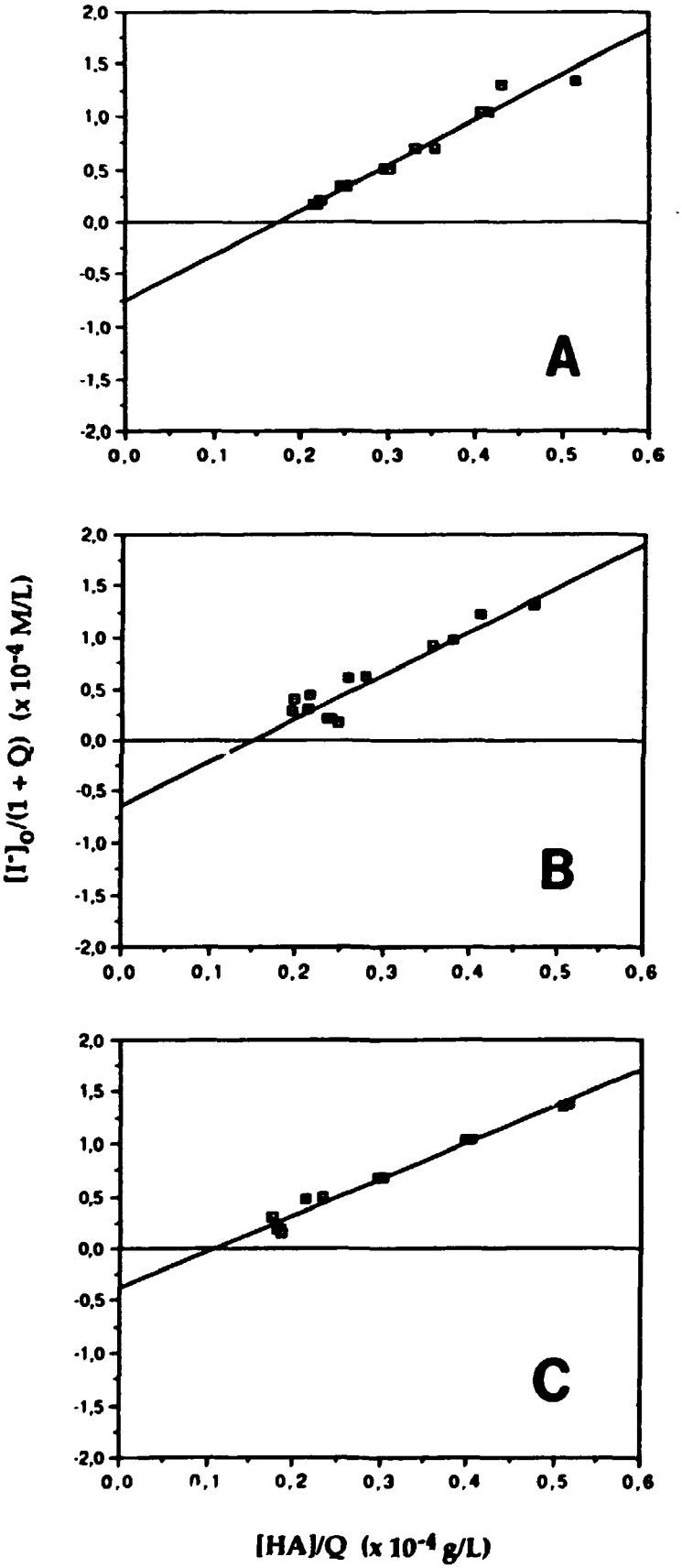
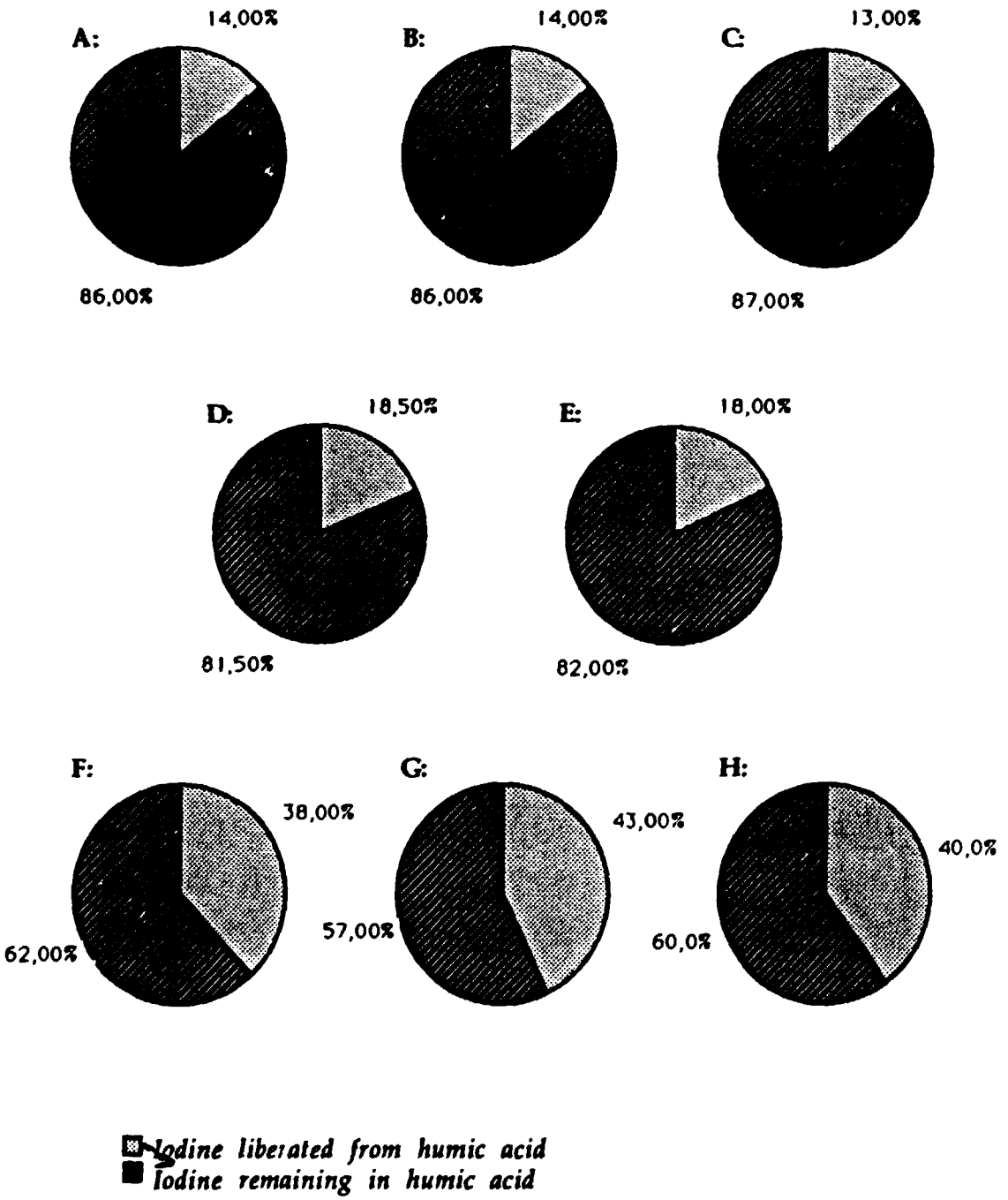


Fig. 2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82,00%
F:
38,00%
62,00%
G:
43,00%
57,00%
H:
40,0%
60,0%
Iodine liberated from humic acid
Iodine remaining in humic acid

Fig. 3

SUMMARY

Iodine appears as number 46 among the more common elements on earth. It is essential for mammals including humans and for that reason it has been subject to investigations which should make the global cycle well understood in whole and in part. Recently, the radioactive isotope of iodine ^{129}I has caught the attention of a number of investigaters. On this background it is intended that this rapport make clearer those fundamental chemical aspects that affect the migration of iodine in soil and groundwater systems.

It has recently been demonstrated that iodine is fixed in the organic fraction of soil and it has been proposed that extracellular enzymes of the peroxidase group are responsible for the fixation.

To gain a basic understanding of the enzyme-catalyzed iodination reaction, phenol and other aromatic compounds were tried; iodination took place by elemental iodine or by iodide in the presence of hydrogen peroxide and peroxidase. It was demonstrated that most of the compounds were iodinated by elemental iodine but only phenol, orcinol and 3,5-dihydroxy benzoic acid were iodinated catalyzed by peroxidases. It was concluded that different aromatic compounds inhibits the function of the enzyme because phenol was not subject to enzyme-catalyzed iodination when these aromatic compounds were present.

Iodination of humic acid was also investigated. It was demonstrated that at least 3 different peroxidases are able to catalyze the iodination of humic acid when iodide and hydrogen peroxide is present. Iodination of humic acid was investigated in detail as the incorporation was measured as a function of different parameters as for instance the concentration of humic acid, iodide and enzyme. A proposal for a mechanism which involves the formation of I_2 and subsequently HOI is given and it is demonstrated that the incorporation of iodine corresponding to different initial ratios of humic acid to iodide may reflect an equilibrium system. In addition, it is demonstrated that isotope

exchange seems to play a crucial role when iodine is present as elemental iodine or as iodide when hydrogen peroxide and enzyme is present.

It is demonstrated that there is a "naturally occurring iodination ability" in soil and that it is extractable by an accepted method for extracting peroxidases from soil. The iodinating ability is heat labile and depends on the presence of hydrogen peroxide. It is proposed that the iodinating ability is physically identical to extracellular peroxidases.

The discussion deals with the kind of organic substructures which can be subject to iodination, the reverse reaction and how the migration of iodine in the terrestrial environment may be affected by the enzyme catalyzed reaction. It is proposed that different types of organic substructures can serve as acceptors and that electrophilic iodine-iodine exchange probably plays the crucial role for the migration of radioiodine under natural conditions.

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Title and author(s)

THE BEHAVIOUR OF IODINE IN THE TERRESTRIAL ENVIRONMENT. AN INVESTIGATION OF THE POSSIBLE ENZYMATICALLY CONTROLLED IODINATION OF HUMIC ACID.

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Abstract (Max. 2000 char.)

Literature on the geochemistry of iodine is surveyed, focusing on fundamental chemical aspects which influence the migration behaviour of iodine in the terrestrial environment. It is stated that the organic fraction in soil plays the predominant role in the retention of iodine. Simple aromatic molecules serve as simple models for humic acid, and humic acid is iodinated catalyzed by haloperoxidases. The enzymatically controlled iodination of humic acid is described in detail and it is demonstrated that the results may reflect a kind of equilibrium. It is shown that soil extracts are able to catalyze the iodination of humic acid and it is suggested that extracellular peroxidases in soil are responsible for the reaction. The enzymatically controlled iodination of humic acid is discussed and some considerations about the influence on the migration of iodine in the terrestrial environment are given.

Descriptors

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